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THE BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY

BY

SIR W. M. BAYLISS, F.R.S.

AND

ARTHUR HARDEN, F.R.S.

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ERRATUM

THE ESTIMATION OF CALCIUM IN BLOOD (LING AND BUSHILL)

The diameter of the centrifuge tube should be 2·6 cm. and not 1·5 as stated in the text and diagram on p. 405.

I. NOTE ON TANNASE.

By DONALD RHIND and FRANCIS EDWARD SMITH.

From the Biochemical Laboratory, Chemical Department, University of Bristol.

(*Received December 14th, 1921.*)

Since the middle of last century, this enzyme has been the subject of many investigations, but it is notable that no method has been described whereby its hydrolysing power may be estimated. The investigation described in this paper was undertaken at the suggestion of Dr Nierenstein, since it was thought that some such method might be of assistance to other work at present in progress in this laboratory.

This enzyme was prepared from *Aspergillus Luchuensis* Inui.[1] by a slight modification of Freudenberg's method [1913]. It was used without further purification, a 1 % solution in distilled water being employed in each experiment.

The gallotannin used was Schering's leviss. puriss. which had been further purified according to Fischer's method [1912]. An approximately 0·3 % solution in distilled water was prepared, and the exact amount of gallotannin present estimated by Nierenstein's caseinogen method [1911] as modified by Spiers [1914].

The hydrolyses were carried out in 250 cc. Erlenmeyer flasks, 100 cc. of gallotannin solution, to which various quantities of tannase solution were added, being employed in each experiment. During the reaction the flasks were plugged with cotton wool, and toluene was added to the reaction mixture to prevent the growth of fungi or bacteria, the whole being kept in a dark incubator at 23°. The amount of gallotannin present in the reaction mixture was estimated immediately after the tannase solution had been added and again after the hydrolysis had proceeded for a certain time.

The details of the estimation are as follows. A solution of approximately 0·1 % $KMnO_4$ was prepared and standardised against pure ammonium oxalate of known strength; 20 cc. of a solution of 0·5 % of indigo carmine in dilute sulphuric acid (A-R reagent, British Drug Houses), being used as indicator. As the indigo carmine reacts with potassium permanganate, it is at first necessary to determine the quantity of the latter used by the indicator solution. To this end 20 cc. of this solution were placed in a large porcelain basin, 750 cc. of distilled water added, and titrated with the $KMnO_4$ solution. The end-point is reached when a faint pink colour can be seen round the edge of the solution. This titration gives the amount of $KMnO_4$ solution taken up by the indicator, which must be allowed for in all calculations. The gallo-

[1] We are indebted to Mr F. W. Mason of the Bureau of Biotechnology at Leeds for a pure culture of this fungus.

tannin present is estimated as follows: 25 cc. of the reaction mixture are withdrawn and 4 cc. of this solution placed in a porcelain dish together with 20 cc. of indigo carmine solution and 750 cc. of distilled water. The whole is then titrated with permanganate solution until the end-point as previously described is obtained. This titration represents both the tannin and non-tannins present. The remainder of the solution is shaken for 15 minutes with 1 g. of fat-free caseinogen and then passed through a barium sulphate filter. This process is repeated twice. When all gallotannin is removed [cf. Nierenstein, 1911], 4 cc. of this filtrate are then titrated as before. The difference between the two titration readings represents the amount of gallotannin present. The actual quantity may be calculated by means of Spier's ammonium oxalate gallotannin ratio, viz. 1 g. of ammonium oxalate is equivalent to 0·4648 g. of gallotannin.

In estimating the amount of gallotannin present after hydrolysis, the procedure is the same as that just described except that it is necessary first to filter off the precipitate of gallic acid formed.

The following table gives the results obtained.

In each case three titrations were made, the mean of these being recorded; also as a check each worker carried out an independent investigation.

Sample	Age in days	Cc. of 1 % tannase solution added to 100 cc. of gallotannin solution	Duration of hydrolysis in hours	Percentage of gallotannin hydrolysed
1	112	10·0	23·75	14·70 14·40
2	2	5·0	19·50	10·52 11·02
2	2	5·0	45·00	16·60 15·70
3	2	5·0	19·50	13·48 13·25
4	2	7·5	19·50	13·00 12·17
4	2	7·5	45·00	18·25 17·30
5	2	10·0	19·50	15·00 15·35
5	2	10·0	45·00	20·00 20·51

From the results given in the above table it is evident that (1) this method represents a moderately accurate procedure for estimating the hydrolysing power of any sample of tannase; (2) the bulk of the hydrolysis takes place during the first 24 hours.

It is proposed by Dr Nierenstein to continue this work at a later date, but since the authors are unable to pursue this research at present, it was thought advisable to publish this preliminary note.

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Fischer (1912). *Ber.* **45**, 923.
Freudenberg (1913). *Ber.* **52**, 177.
Nierenstein (1911). *Chem. Zeitg.* **36**, 31.
Spiers (1914). *J. Agric. Sci.* **6**, 77.

II. THE ACTION OF HYPOPHYSIN, ERGAMINE AND ADRENALINE UPON THE SECRETION OF THE MAMMARY GLAND.

By ERNEST ROTHLIN, ROBERT HENRY ADERS PLIMMER and ALFRED DENNIS HUSBAND.

From the Biochemical Department of the Rowett Research Institute of Animal Nutrition, University of Aberdeen and the North of Scotland College of Agriculture, and the Physiological Institute, University of Zurich.

(*Received December 20th, 1921.*)

The action of extracts of various organs, especially that of the pituitary gland (hypophysin) upon milk secretion has been frequently investigated. The results of these investigations have been summarised by Hammond [1913], who made an extensive study of the action of hypophysin upon the milk flow in the goat. He found that there was an immediate action and that the effect soon passed off, and he concluded that the action was not upon the muscular tissue of the gland but rather upon the glandular epithelium. The action depended upon the state of nutrition of the animal and upon the stage of lactation. The composition of the milk was not appreciably altered, though that produced by the action of the drug had a higher percentage of fat than other samples. The milk analyses were limited to estimations of fat and total solids and did not extend over long periods.

Hammond and Hawk [1917] added some further details respecting the action in different states of nutrition and tested adrenaline, which was found to have no effect.

As two goats under normal conditions, on a constant diet, but in different stages of lactation were available, there arose the opportunity of once more testing the action of hypophysin upon the stage of lactation and of extending Hammond's results. Complete analyses of the milk of these goats had been previously made, and by continuing this procedure and including extra samples some further details could be ascertained. At the same time it was of interest to compare its action with that of adrenaline and of ergamine or histamine. The latter substances, as is well known, have an action corresponding with that of excitation of the sympathetic or para-sympathetic nervous systems. Their action, if any, would give probably some information upon the innervation of the mammary gland with nerve fibres of the involuntary nervous system. Hammond's results with hypophysin have been confirmed and extended; ergamine and adrenaline, as might have been expected, had no action.

Experimental.

The hypophysin, ergamine as acid phosphate, and adrenaline used for injection were the commercial preparations supplied by Burroughs, Wellcome and Co. The injections were always subcutaneous in the lumbar region of the body. They were made at different times of the day, usually from one to two hours before milking and generally in both goats about the same time. Before the injection of the drugs an injection of 0·9 % saline was made and once again later; it had no effect and thus the effect of the actual injection was excluded.

The experiments were carried out in two periods; during the first period there was an interval of only one or two days between the injections; during the second period there was an interval of about seven days between the injections of the three drugs in order to ascertain if the total volume of the milk differed in the period of injection from that before and after. The injections were then made more frequently, sometimes twice in the day.

The two goats used in these experiments were in different periods of lactation. Goat *A* had two kids on June 14th, goat *N* one kid on Feb. 2nd, 1920. Goat *A* was in a good state of lactation, goat *N* in a poor state.

Goat *A* during the first ten days was outdoors at grass by day and indoors by night; subsequently she was kept indoors permanently, fastened by a chain in a ground floor room of the building. Goat *N* was always kept in the building in the same room, but in a cage in which she could freely move and turn about. Both goats were given 112 g. of oatmeal at about 8 a.m., noon and 5 p.m. This was eaten rapidly. Except for the first ten days, when goat *A* had grass during the day, after the oatmeal both had 454 g. of hay, which they were allowed to eat at their leisure. They were thus under fairly normal conditions and upon a constant diet. Their weights varied very little during the course of the experiment which lasted about three months, and as far as could be noticed they were in excellent health.

The milk was not drawn by suction, but always by hand by the same experienced milker, who had drawn the milk previously over a long period. During this time the goats were milked twice daily, morning and evening; the two samples were mixed and analysed. To observe the effect of the drugs it became necessary to draw the milk more frequently and it was taken at 9.30 a.m., 11.30 a.m. and 5 p.m. and occasionally at 6.30 or 7 p.m., depending upon the time of injection.

Complete daily analyses of the milk of these goats had been previously made. Owing to the number of samples now taken (six to eight) it was not possible to analyse each separately and two or more samples were combined. The combination of two samples was made in such a way that the particular sample after injection was kept separate. The analyses are shown in the table, numbered 1, 2 or 3 according to the milking time of 9.30 a.m., 11.30 a.m. and 5 p.m.

The methods of analysis were those usually employed in milk analysis. Fat was estimated by Soxhlet's method, total protein by Kjeldahl's method: caseinogen by this method after its precipitation from the sample by dilute acetic acid and washing; albumin in the filtrate from the caseinogen by precipitation with tannic acid, washing and Kjeldahl's method. The nitrogen figures by Kjeldahl's method were multiplied by the factor of 6·38 to give the protein. Lactose was estimated in a sample, precipitated by "dialysed iron," by Fehling's method. Ash was determined by incineration in a small crucible. The data are expressed in grams per 100 cc. milk.

The figures in the following table show the volumes at each milking, and the fat analyses, and the calculations to give the figures for the output of the whole day, the times of injection, etc. The data are so numerous that they are greatly abbreviated, and only those showing the essential features are given. The amounts of lactose, total protein, caseinogen and albumin except on the first two days are omitted and none of the figures of the second period is shown.

Results.

(*a*) *Changes of Volume.*

1. *Hypophysin.* The injection of hypophysin generally about two hours before the milking time produced a flow of milk only in goat *A*, which was in an early stage of lactation. The effect is distinctly marked in the volume of the second sample collected after an interval of two hours from the first sample. The volumes of 250 and 260 cc. were obtained in comparison with normal volumes varying from 35 to 145 cc. The volume of the next milking was then smaller than the normal, 230 and 150 cc. against 255 and 325 cc. An increase was not definitely noticed in the volume of the third sample collected after an interval of about six hours from the previous one. A volume of 350 cc. was obtained between volumes of 325 and 410 cc., but the average volume for this milking time was between 200 and 300 cc. No increase in volume was observed in the first sample collected at 9.30 a.m., *i.e.* about 16 hours after the sample at 5 p.m. The total daily volume of milk was not appreciably altered. A change over the total period could not be observed as the goat *A* gave a gradually diminishing volume of milk.

No change in volume of the samples was observed in goat *N* after an injection of hypophysin. This goat gave a more regular daily volume and the effect was to make a slight general increase of the total volume; previous to the injection the average volume was 423 cc.; during the period it was 448 cc. and after 468 cc. A similar slight increase was again noted later, the volumes being 395, 424 and 509 cc. The increase may however be due to other circumstances, as the goat *N* had at the time of this injection been in lactation for eight to nine months. The injection in goat *A* at the later period did not produce a rapid flow as at the former time, but there was a very slight increase of total volume; the averages of the periods were 710, 724, 730.

Protocols.

Date Sept.		Goat *A* Vol.	Ash	Fat	Lact.	T. prot.	Cas.	Alb.	Goat *N* Vol.	Ash	Fat	Lact.	T. prot.	Cas.	Alb.
7		1100	0·83	6·22	4·06	3·64	3·00	0·47	450	0·91	5·75	4·55	3·57	2·57	0·75
8		1200	0·80	7·80	4·23	3·59	2·95	0·48	390	0·90	5·07	4·64	3·73	2·75	0·75
*9		1200	0·76	7·02	4·26	3·61	2·95	0·51	430	0·97	5·50	4·60	3·52	2·53	0·77
†10	1	900	0·85	4·20	4·36	3·77	2·90	0·51	315	0·91	5·31	4·60	3·63	2·53	0·75
	2	250	0·80	11·21	4·00	3·61	2·75	0·46	10	—	12·07	4·37	3·68	2·50	0·80
	3	230	0·82	9·12	4·11	3·85	2·90	0·47	140	0·90	6·25	4·64	3·69	2·59	0·70
per total		1380	0·83	6·29	4·26	3·75	2·87	0·49	465	0·91	8·47	4·61	3·65	2·55	0·74
‡11	1	900	0·87	5·75	4·31	4·00	3·07	0·53	308	0·91	5·22	4·70	3·77	2·64	0·75
	3	215							90						
	2	55	0·80	11·35	4·08	3·78	3·02	0·47	12	—	8·67	4·38	3·73	2·26	0·52
		1170	0·87	6·01	4·30	3·99	3·07	0·53	410	0·91	5·28	4·66	3·77	2·63	0·74

* Both goats injected with 1 cc. 0·9 % NaCl after first milking.

† Both goats injected with 0·5 cc. hypophysin after first milking; milk collected one hour after injection (10.25 a.m.).

‡ No injection.

Date Sept.		Goat *A* Vol.	Ash	Fat	Goat *N* Vol.	Ash	Fat	Remarks
12	1	920	—	—	310	—	—	No injection.
	2	100	—	—	35	—	—	
	3	255	—	—	85	—	—	
		1275	0·84	7·80	430	0·90	4·99	
13	1	840	0·83	5·00	300	0·91	4·89	Both injected with 1 cc. hypophysin after first milking; milk collected one hour after injection.
	2	260	0·74	14·09	37	0·82	9·29	
	3	150	0·90	7·37	83	0·94	4·77	
		1250	0·82	7·09	420	0·91	5·25	
14	1	940	0·85	4·13	335	0·96	4·31	No injection.
	3	325			118			
	2	95	0·74	10·35	42	0·90	10·74	
		1360	0·84	4·57	495	0·95	4·86	
15	1	680	0·86	4·65	325	0·92	4·50	Goat *A* injected with 1 cc. 0·9 % NaCl at 3 p.m. Goat *N* injected with 1 cc. hypophysin at 3 p.m.
	2	70	0·86	10·62	10	—	9·65	
	3	350	0·82	9·00	130	0·90	6·29	
		1100	0·85	6·41	465	0·91	5·11	
16	1	520	0·84	4·00	280	0·96	4·10	No injection.
	2	95	0·80	6·30	40	0·90	7·52	
	3	410	0·84	6·39	115	0·96	4·69	
		1025	0·84	5·37	435	0·95	4·57	
17	1	800	—	—	305	0·94	3·89	Both goats injected with 1 cc. hypophysin at 8.15 a.m.; milk collected one hour later.
	2	145	—	—	45	—	5·98	
	3	265	—	—	95	0·92	4·00	
		1210	0·83	5·54	445	0·93	4·12	
18	1	750	—	—	325	0·95	4·12	No injection.
	2	35	—	—	40	0·88	7·40	
	3	210	—	—	110	0·88	4·69	
		995	0·81	5·51	475	0·93	4·53	
19	1	680	0·80	5·15	315	—	—	No injection.
	2	50	0·80	9·30	35	—	—	
	3	230	0·80	8·40	110	—	—	
		960	0·80	6·15	460	0·95	4·15	
	4	30	0·83	9·40	35	0·94	5·53	
20	1	660	0·85	5·50	300	—	—	Goat *A* injected with 1 cc. hypophysin at 8.15 a.m.; milk collected one hour later.
	2	65	0·82	8·10	30	—	—	
	3	175	0·88	6·52	105	—	—	
		930	0·85	6·00	470	0·94	4·39	
21	1	620	0·87	5·82	305	0·93	3·94	Goat *N* injected with 1 mgm. ergamine phosphate at 9.45 a.m.; milk collected one hour later.
	3	145			45	0·75	8·21	
	2	70	0·82	10·25	100	0·94	4·97	
		835	0·87	6·19	450	0·93	4·60	

Date Sept.		Goat *A* Vol.	Goat *A* Ash	Goat *A* Fat	Goat *N* Vol.	Goat *N* Ash	Goat *N* Fat	Remarks
22	1	570	0·85	5·60	250	0·94	4·36	Goat *A* injected with 1 mgm. ergamine phosphate at 9.45 a.m.; milk collected one hour later.
	3	120	0·88	6·30	100			
	2	110	0·74	9·38	35	0·90	7·45	
		800	0·84	6·23	385	0·94	4·64	
23	1	730	0·90	5·72	320	0·94	4·83	Both goats injected with 1 mgm. ergamine phosphate at 3.45 p.m.; milk collected at 5 p.m.
	2	140	0·82	10·02	18	—	7·82	
	3	160	0·91	5·80	142	0·90	5·26	
		1030	0·89	6·41	480	0·93	5·07	
24	1	660	0·85	5·15	315	0·92	4·73	No injection.
	2	80	0·83	9·22	30	0·86	9·85	
	3	200	0·86	6·50	105	0·93	4·95	
		940	0·86	5·78	450	0·92	5·12	
25	1	640	0·90	6·05	300	0·92	4·56	Goat *A* injected with 0·5 mgm. ergamine phosphate at 8.15 a.m. Goat *N* injected with 1·0 mgm. ergamine phosphate at 8.15 a.m. Milk of both goats collected one hour later.
	2	50	0·80	9·54	20	—	8·54	
	3	140	0·87	7·40	100	0·91	5·18	
		830	0·89	6·49	420	0·92	4·90	
26	1	570	—	—	295	—	—	
	2	70	—	—	15	—	—	
	3	205	—	—	120	—	—	
		845	0·89	6·73	430	0·98	4·96	
	4	33	lost	7·43	33	0·93	6·57	
27	1	622	0·88	5·03	282	0·94	4·78	Both goats injected with 1 mgm. ergamine phosphate at 3.30 p.m. Milk collected at 5 p.m.
	2	20	—	—	10	—	—	
	3	225	0·87	7·04	130	0·96	6·46	
		900	0·88	5·67	455	0·94	5·40	
28	1	600	0·87	5·68	300	0·92	4·67	Both goats injected with 1 mgm. ergamine phosphate at 6.15 p.m. Milk collected at 9.15 a.m. next day.
	2	50	—	—	15	—	—	
	3	130	0·86	7·32	125	0·93	7·94	
		780	0·87	6·66	440	0·92	5·71	
29	1	630	0·88	7·64	295	0·95	5·15	No injection.
	2	30	0·86	8·76	10	0·93	5·72	
	3	220			130			
		880	0·87	7·96	435	0·94	5·33	
30	1	540	0·87	6·80	295	0·93	5·62	Both goats injected with 1 mgm. adrenaline at 1.15 p.m.; milk collected at 5 p.m.
	2	45	—	—	20	—	—	
	3	110	0·87	7·63	100	0·93	8·01	
		695	0·87	7·21	415	0·93	6·31	
Oct.								
1	1	620	0·90	6·92	285	0·97	5·28	Both goats injected with 1 cc. 0·9 % NaCl at 1.30 p.m.; milk collected at 5 p.m.
	2	40	—	—	35	—	—	
	3	210	0·86	8·02	110	0·94	6·51	
		870	0·89	7·24	430	0·96	5·69	
2	1	590	0·81	5·63	300	0·93	5·00	Both goats injected with 1 mgm. adrenaline at 9.45 a.m.; milk collected at 11 a.m.
	2	30	—	—	15	—	—	
	3	200	0·86	8·28	100	0·91	7·01	
		820	0·84	6·37	415	0·92	5·56	
	4	24	—	9·24	20	—	8·34	
3	1	536	0·87	6·60	265	0·94	5·46	No injection.
	2	40	—	—	35	—	—	
	3	140	0·87	8·38	100	0·94	6·66	
		740	0·87	7·12	420	0·94	5·98	
4	1	650	0·86	5·61	315	0·91	4·87	Both goats injected with 1 mgm. adrenaline at 1.15 p.m.; milk collected at 5 p.m.
	2	30	—	—	10	—	—	
	3	170	0·88	8·90	110	0·92	6·21	
		850	0·86	6·27	435	0·91	5·24	
5	1	480	0·91	6·00	260	0·95	4·75	No injection.
	2	75	—	—	10	—	—	
	3	190	0·89	7·49	115	0·94	5·70	
		745	0·90	6·53	385	0·95	5·06	

2. *Ergamine.* The injection of ergamine had no pronounced effect on the separate volumes of the samples of either goat and the average volume of the total milk per day was about the same as when no injection was made. A general effect over the whole period was not noticeable in the case of goat *A* which was giving a diminishing flow of milk, but a slight diminution can be made out in the case of goat *N*. During the second period of injection the flow of milk in goat *A* was more constant, the average daily volumes being 645, 656, 657 cc. for the pre-, actual, and post times; the average figures of goat *N* at this time were 532, 493, 576. In both cases the changes are not essentially different from the ordinary daily volumes.

3. *Adrenaline.* During the first period no effect could be seen in the case of either goat, both the separate volumes and the average daily volumes remaining unchanged. During the second period there was an apparent increase in goat *A*, but the increase occurred at a time when the daily flow was lessening; the reverse effect can be made out in goat *N*. The general effect is not sufficient to indicate any definite action of adrenaline.

(*b*) *Composition of the Milk.*

No real change in the chemical composition of the total milk per day occurred in either goat after the injections of hypophysin, ergamine and adrenaline. If any change did occur, it was not greater than the normal variations in composition. These normal daily variations are most marked in the fat content. Possibly the fat content was lowered over the total period during which hypophysin was injected and raised during the ergamine period, but again the alterations were not more than those which occur daily.

The three separate samples of milk taken at different times of the day after different intervals had normally a distinctly different composition. The fat content was always highest in the second sample taken two hours after the first, and the early morning sample had the lowest amount of fat; it was taken after an interval of 16 hours. The higher fat content was observed in both goats and is thus independent of the period of lactation. The second sample had less protein and lactose than the other samples. On the average the first sample at 9.30 a.m. had more protein and lactose than the third sample, but the differences are not distinctly marked off.

If allowance be made for the ordinary daily variations, the separate samples do not show any appreciable difference from the normal.

Discussion.

Since a marked increase in the volume of milk was only observed in goat *A* at the time of the second milking, and since it was followed by a diminished volume at the third milking, it appears that the action of hypophysin is powerful, rapid and of short duration. The phase of hyper-secretion after injection is followed by a compensatory phase of hypo-secretion. This effect

explains the absence of an increase in volume at the time of the first milking and third milking. The interval between the milkings was here 16 hours; an increase followed by a decrease would not be noticed. The interval between the first and second milkings was two hours. The gland secretes normally (Sept. 12) about 50 cc. every hour; after hypophysin the secretion was 125 cc. (Sept. 10) and 130 cc. (Sept. 13) per hour. Hypophysin had no effect in goat *N* and at the later period in goat *A*. The action of hypophysin is therefore probably not directly upon the gland. If the smooth muscle were stimulated, a flow of milk should follow at either period of lactation. If the secreting cells were affected a flow of milk should also follow in any case. It is most probable that it may act indirectly through the reproductive organs, which have been proved to contain substances acting upon the secretion of the mammary gland. At the earlier period of lactation the reproductive organs will be in a state of activity, whereas in the later stage their state of activity will have disappeared. Hypophysin thus may act upon an active organ which produces the galactogogue; further work will be required to determine whether this active organ be the ovary, corpus luteum, uterus, or placenta.

In practice, the injection of hypophysin will only be of value at early stages of lactation and it may be able to bring a gland into activity when it is not already in that state. It must be remembered that the total volume produced by the gland is not appreciably increased, as shown above and previously described by other investigators.

The composition of the total milk per day is not altered by the injection of hypophysin. It has been previously considered that hypophysin causes a flow of milk with a higher fat content, but, as the data show, it is followed by a milk with a lower fat content. The high fat content of a sample taken at a short interval between milkings is normal and is not really due to the action of hypophysin.

As milk contains more fat if collected at a short interval after a previous longer interval, it appears that the secreting mechanism of the mammary gland is of a two-fold character; a mechanism producing fat and a mechanism producing protein and lactose. The fat-producing mechanism begins to act first and a milk of high fat content results; the protein and lactose mechanism acts later and dilutes the fat content to the normal value. The fat mechanism is probably more easily influenced by other conditions, since the amount of fat in milk is very variable, whilst those of protein and lactose are fairly constant.

Former investigators have observed that adrenaline has no action upon the mammary gland; these results confirm their work.

Ergamine also has no action. If these substances have a positive or negative action, it is rapidly followed by compensation.

The inaction of adrenaline points to the absence of sympathetic nerve fibres from the mammary gland, if the general law of the relationship between the action of adrenaline and the presence of sympathetic fibres is here correct.

It has been suggested from analogy to other glands (stomach) that ergamine perhaps acts on para-sympathetic fibres. The inaction points to the absence of such fibres from the mammary gland.

SUMMARY.

Hypophysin produces a flow of milk only in the early stages of lactation. Its action is rapid and powerful, but of short duration. The flow (hyper-secretion) is followed by a smaller quantity than normal (hypo-secretion). The total volume of milk per day is not altered.

Since hypophysin does not act at later stages of lactation, it is probable that its action is indirect through the organs of reproduction.

The quality of milk secreted after the action of hypophysin is not different from the normal. Normal milk has a high fat content, if it be collected at a short interval after the last milking.

The secretory activity of the mammary gland is not influenced by the subcutaneous injection of adrenaline or ergamine. The gland probably does not contain sympathetic or para-sympathetic nerve fibres for the secretory mechanism. If they be present, the inaction of adrenaline and ergamine is exceptional.

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III. THE REARING OF CHICKENS ON THE INTENSIVE SYSTEM. PART I. THE VITAMIN REQUIREMENTS.

PRELIMINARY EXPERIMENTS.

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The attempts of previous workers to rear chickens in confinement under laboratory conditions have not been altogether successful. Drummond [1916] considered that there were other factors than an adequate diet which must be supplied to the growing chick. Osborne and Mendel [1918] commented upon various statements by Drummond and remarked "if the conditions under which chickens continue to grow normally in confinement can be learned, it will be possible to obtain much information of practical use in poultry husbandry." Further they say "There is a widespread belief among poultry raisers that young chickens cannot be reared under the artificial conditions of housing and diet which many other experimental animals tolerate without detriment. The current ideas are expressed in the statement that the birds must be kept 'on the ground,' that they must have exercise, outdoor life, and green food."

In their previous experiment [1916] with four chicks from 3 to 4 weeks old, two died and two were observed to the age of 77 days. Two other birds 81 days old at this time were actually kept for a total of 309 days in which time they reached maturity in apparent full vigour. The birds at the ages of 77 and 81 days had very ruffled feathers; this appearance was attributed to frequent handling. Osborne and Mendel believed the success was largely due to the supply of blotting paper as roughage. Out of another group of ten birds, two only reached maturity; the others failed to grow and showed leg weakness. We may record the better success of Buckner, Nollau and Kastle [1915] and of Palmer and Kempster [1919]. Hart, Halpin and Steenbock [1920] have emphasised the frequent occurrence of leg weakness in chickens reared in confinement and, like Osborne and Mendel, attribute the cause to

the absence of roughage from the diet. Drummond's birds were fed upon ordinary commercial chicken food; Osborne and Mendel fed their birds upon the foods which they supplied to rats, containing butter fat and protein-free milk as sources of the A and B accessory food factors, a diet quite satisfactory for their growth. Buckner, Nollau and Kastle used grain mixtures and supplied some "green" food. Hart, Halpin and Steenbock used a diet similar to that of Osborne and Mendel.

It is now known that, though rats and a few other animals can be reared on a diet containing only A and B factors, most animals, including man, require the inclusion of C factor for the maintenance of health. It was thus possible that the poor success was due to the absence of this third factor, although it was added to the diets fed by Hart, Halpin and Steenbock. With reference to this third factor, the work of Chick and her colleagues [1919] insists upon the inclusion of a definite daily quantity which must be above a certain minimal amount. The necessity for a daily amount of the other vitamins has not been so clearly established, but at any rate it is indicated by the experiments of Cooper [1913] and of Chick and Hume [1917].

In these experiments the birds have been supplied with the usual protein, carbohydrate and fat; salts other than those contained in the foodstuffs were not added. Accessory food factors have been given in the form of cod liver oil for A, autolysed yeast or marmite for B, and lemon or orange juice for C. The choice of these three substances was made on account of the general experience that cod liver oil is the most concentrated in A factor, marmite in B, and lemon or orange juice in C, as well as the fact that these substances are very fairly constant in their respective vitamin content. The diet was composed essentially of oatmeal and milk and no account was taken of the presence of vitamins in these foods. Water was always freely provided.

Arbitrary amounts of the vitamin foods had to be chosen as the requirements for chickens are unknown. Later work is designed to determine the minimal daily amount of each vitamin, and subsequently these amounts will have to be translated into the terms of natural foodstuffs as supplied by the poultry keeper. It must be pointed out that the natural foods are very unequal in their vitamin content and that at present, in the absence of chemical knowledge of vitamins, only comparable data are capable of investigation. Also, it must be clearly stated that the diet now supplied is not one that would be given under ordinary conditions to poultry.

With the food supplied 21 chickens out of 24 have been reared to maturity; two were lost from illness apparently due to insufficient vitamin B in the diet and one was lost accidentally; in other terms 91 % were successfully reared. In Part II [1922] it will be seen that nine birds out of nine, or 100 % were brought to maturity.

Experimental.

Housing. The chicks up to the age of about four weeks were housed in an ordinary type of foster mother, heated by a paraffin lamp[1]. After this age they were kept in wooden poultry houses measuring $6 \times 4\frac{3}{4} \times 6\frac{1}{2}$ feet high with an entrance door at one end and an opening into a run at the other end. Ventilation hatches were provided at each end at the top. The run of wire netting on wooden supports on a wood floor had the size of $6 \times 4\frac{3}{4} \times 3$ feet high. Altogether there were four identical houses, two on the roof of the building, two in a room measuring $30 \times 20 \times 15$ feet high on the ground floor of the Institute. The foster mother was kept in the ground floor room.

The wooden floors of the foster mother, poultry houses and runs were covered with broken peat moss litter, except that during wet weather, sand and coal ashes were used in the houses on the roof. The peat moss litter was scraped about but, as far as could be ascertained, very little was eaten. Grit was supplied in the form of coal ashes from the age of about two months. The birds were weighed separately every two days.

Diet. There is very little information about the daily quantity of food consumed by little chicks; it is stated [Board of Agriculture and Fisheries, 1907] that chicks eat about 3 lbs. of dry food during the first eight weeks of their life. Consequently definite amounts were prepared every day; portions were given at intervals of 2–3 hours to the little chicks and 2–4 times daily as they grew older. In this way a record could be kept of the amount eaten at the different ages. It was found that the total consumption corresponded to that stated.

Kilned oatmeal of pin head size was selected as the basal diet, mainly on account of its method of manufacture which would exclude the presence of vitamins, but their entire absence could not be expected. A small quantity of milk was also given to provide extra protein; the amount was at first rather variable, but later was always measured. Towards the end caseinogen was substituted for milk in the case of half the birds and at the same time B factor was excluded, since a comparison between caseinogen and marmite and at the same time an estimate of the vitamin content of the caseinogen, were wanted.

The accessory food factors were added to the daily quantity of oatmeal. According to the health of the birds the original amounts required alteration. The course of the experiment is most conveniently divided into periods from which the variations in food consumption and vitamin needs are more easily ascertained.

Twenty-four chicks were comprised in the group, 12 white and 12 black Leghorn. They were hatched in an incubator and were from Miss Fraser's strains at the North of Scotland College of Agriculture. At the time of their arrival their age was approximately three days; several were suffering from diarrhoea and were very weak and some mortality was expected.

[1] Kindly lent by Miss Fraser of the North of Scotland College of Agriculture.

Period 1 *from July* 13*th to Aug.* 10*th*, 1920. The birds were housed in the foster mother and supplied daily with 120 g. oatmeal, 5 cc. cod liver oil, 0·5 g. marmite, 30 cc. lemon juice.

Extra food, as required, after the above mixture had been eaten, was given in the form of dry oatmeal, or a mixture of dry oatmeal and biscuit meal; at first 30 g. was eaten daily, but it gradually increased until at the end of the period 200 g. was eaten. Including the basal mixture, the total food consumption per bird increased from 6 to 13 g. per day.

In addition the birds were given 200 to 250 cc. of milk every day; it was mostly spilt and thus no record of its consumption could be made. On two occasions the yolk of an egg was given and lemon skins were put into the run for the birds to peck at and for their amusement; as far as could be seen, none was eaten.

On July 21st, *i.e.* on the ninth day, one of the weak birds was found dead in the outer part of the foster mother.

The other chicks recovered from the chicken diarrhoea and no signs of illness were noticed in any of the other birds.

The average weight of the birds on July 27 was 78 g.; on Aug. 20 it was 150 g. The increase averaged 5·1 g. per day.

Period 2 *from Aug.* 11*th to Sept.* 3*rd*, 1920. The 23 birds were now divided into two groups. Group I had six black and six white; group II had five white and six black chicks. The selection was made so that the average weight of the two groups was approximately the same: group I weighed 154, group II 156 g. per bird. The two groups were housed in the poultry houses in the ground floor room.

Each group received as basal diet 120 g. of oatmeal. The vitamins were added as before, but the amounts were divided between the two groups, except in the case of C factor. Each group received 2·5 cc. of cod liver oil and 0·25 g. of marmite per day. The difference in the amount of C factor was made on account of the stress that poultry keepers lay on green food for birds, and because, presumably, C factor is the most abundant in these foods. Group I was given 15 cc., group II 20 cc. of lemon juice per day; in the case of group I it was thus the same as before.

For the first three days extra food was given as oatmeal and biscuit meal; each group consumed about 44 g. per day. On Aug. 14th, the extra food was given as a mixture of oatmeal and milk, 10 cc. of milk being mixed with 30 g. of oatmeal. The proportion of milk was raised to 20 cc. on Aug. 26th, since the protein ratio was very low on the former mixture. The protein ratio of the total thus became 1 : 6 approximately. No account of the presence of vitamins in the milk was taken as it is now proved that the vitamin content of milk is variable and the quantity so supplied was expected to make little difference to that supplied directly.

Five days after the separation into two groups, on Aug. 15th, illness was observed amongst the birds of group I. There was no particular characteristic

sign; the birds were less active and drooped. The cause was attributed to insufficiency of C factor. The quantities were therefore increased. Group I was given 35 cc. and group II 30 cc. per day, *i.e.* in the reverse order to that previously. A little improvement was noticed, but the birds still remained unwell. Consideration was then given to the amount of B factor. The quantity of 0·25 g. to each group was very small and the amount was raised to 0·5 g. per day to each group on Aug. 19th.

After this increase some birds of group II showed similar signs of illness to those of group I, and the quantity of lemon juice was again increased to 40 and 50 cc. per day respectively to groups I and II.

Group H. One bird of group I was so ill on Aug. 24th that it was removed and housed in the foster mother (not heated). The next day two other birds from group I and one bird from group II were also put into the foster mother. This so-called hospital group H was given the basal diet of oatmeal and vitamins supplied to group II, but with the cod liver oil increased to 5 cc.; so far no alteration had been made in the quantity of A factor and the illness was possibly due to its insufficiency. The increase had no effect; the birds got no better and on Aug. 27th the first bird of group I died. Its death was diagnosed by Dr W. Taylor as due to chicken cholera.

Some of the birds now showed distinct signs of leg weakness, drooping wings and ruffled feathers. These symptoms have been described in the case of birds suffering from polyneuritis gallinarum in its early stages. The cause of the illness was thus indicated to be due to insufficiency of B factor. Extra marmite was consequently given, but owing to the poor appetite of the birds it could not be given in the food; on Sept. 1st, it was therefore supplied in the form of a 2 % marmite drink. This was greatly relished and on the third day unmistakable signs of improvement in health were observed. Just as this improvement was noticed two more birds from group II had to be admitted to the hospital group (Sept. 3rd). With the marmite drink they also got better in three days. The drink was stopped on the 9th Sept. and as the birds were now quite well they were returned to their groups on Sept. 11th.

Period 3 *from Sept. 4th to* 10*th*, 1920. The birds remaining in groups I and II were supplied from Sept. 1st to 3rd with the same daily diet consisting of 300 g. oatmeal, 120 cc. milk, 2·5 cc. cod liver oil, 0·5 g. marmite, 50 cc. lemon juice. Since there was no object in continuing the same diet to both groups, a change was made on Sept. 4th in the quantity of A factor. Group II was given 10 cc. Loss of appetite was noticed in both groups and several more birds in group II were ill. A drink of lemon juice had no effect. The remarkable effect of the marmite drink had just been observed in group H and it appeared clear that too little B factor was being supplied. The quantity of marmite in the basal diet of both groups was therefore doubled on Sept. 6th. Group I relished the increase by showing an improved appetite, but group II still appeared ill. On Sept. 8th group II was given a drink of 2 % marmite; its effect was again striking. It thus appeared clear that too little B factor had

been given and that chickens are very susceptible to an insufficiency; it was very noticeable that the leg weakness disappeared with the increase. Guinea-pigs show a similar behaviour, but their susceptibility is to C factor. The daily amount of marmite in the diet was doubled for the last two days of this period.

Period 4 *from Sept.* 11*th to* 16*th*, 1920. On Sept. 11th the birds of the hospital group were returned to their respective groups. On account of the death each group had now eleven birds.

The effect of the cod liver oil on group II had had the noticeable effect of producing a loss of appetite and signs of ill-health; another period of a similar diet was therefore tried. Both groups were given more marmite, 2·5 g. per day and the same amount of lemon juice 30 cc. gradually reduced from 50 cc. Oatmeal remained as before. Group I had 5 cc. of cod liver oil; group II 12·5 cc. The birds of group I (except one which was given 0·5 g. marmite, dissolved in water, by hand on Sept. 14th, after which it recovered) appeared well and had a good appetite. The birds of group II showed marked loss of appetite, and the quantity of marmite was doubled on Sept. 14th; by Sept. 17th their appetite had again become normal. The extra cod liver oil was thus not itself the cause, but rather the extra fat required B factor for its assimilation. Braddon and Cooper [1914] have made similar observations in cases of beri-beri, and have suggested that the amount of B factor ran parallel to the caloric value of the diet.

During this period a bird of group I swallowed a nail and died.

Period 5 *from Sept.* 19*th to Nov.* 10*th*, 1920. The above observations indicating that the amount of B factor needed bore a constant ratio to the amount of carbohydrate and fat in the diet led to a general rearrangement of the diet. For every 30 g. of oatmeal a quantity of 0·5 g. of marmite was given; cod liver oil and lemon juice were given in a constant quantity per day. The only difference in the two groups was the extra cod liver oil for group II and its balancing by 2 g. of marmite. The daily food consumption for the first part of this period was the following:

	Group I				Group II				Extra for groups I and II		
	Oatmeal g.	Cod liver oil cc.	Marmite g.	Lemon juice cc.	Oatmeal g.	Cod liver oil cc.	Marmite g.	Lemon juice cc.	Oatmeal g.	Marmite g.	Milk cc.
Sept. 19–21	180	5	3	30	180	12·5	5	30	120	2	80
,, 22–Oct. 17	180	5	3	30	180	12·5	5	30	240	4	160
Oct. 18–21	180	5	3	30	180	12·5	5	30	360	6	240

The food was supplied in two portions, and in consequence of the greater size of the birds the milk portion was rapidly increasing above the original quantity. It was therefore decided to reduce the milk to 100 cc. per day and for greater convenience all the items were made into a single mixture:

	For group I					For group II				
	Oatmeal g.	Milk cc.	Cod liver oil cc.	Marmite g.	Lemon juice cc.	Oatmeal g.	Milk cc.	Cod liver oil cc.	Marmite g.	Lemon juice cc.
Oct. 22–Nov. 10	660	100	5	11	30	660	100	12·5	13	30

These quantities were eaten daily by the birds of group II, but on two occasions they were not eaten by group I, due to the fact that this group had 10 birds against 11 in group II.

There were no signs of illness throughout this period of 54 days; the birds grew steadily, increasing on the average 25 g. every two days. Group I increased from 406 to 1019 or 613 g., group II from 329 to 1011 or 682 g. It would appear that the extra cod liver oil had a stimulating action, but as two birds of group II were small at the start and two were cockerels of a large strain, it was not a real effect.

An alteration in the housing was made on Oct. 12th, both groups being moved to the roof. The move made no difference to the regular weight increase nor to the daily food consumption, so that housing is of minor importance provided diet and cleanliness are satisfactory. During this period the cocks began to crow, the first one crowing on Oct. 13th.

Period 6 *from Nov.* 11*th to Jan.* 13*th*, 1921. The quantities of the several accessory factors supplied in the previous period were evidently sufficient to keep both groups in perfect health. Group III (see Part II) was receiving a diet containing caseinogen, secwa and 0·25 g. marmite per 30 g. oatmeal per day, *i.e.* half the amount of marmite. The caseinogen and secwa would thus together contain 0·25 g. of B factor reckoned as marmite. It was possible that the caseinogen alone contained B factor equivalent to that in the marmite. Caseinogen was therefore substituted for marmite in group I. It was also of interest to compare the protein of caseinogen with that contained in marmite. The diets thus became:

	For group I					For group II				
	Oatmeal g.	Milk cc.	Caseinogen g.	Cod liver oil cc.	Lemon juice cc.	Oatmeal g.	Milk cc.	Marmite g.	Cod liver oil cc.	Lemon juice cc.
Nov. 11–Dec. 6	660	100	13	5	30	660	100	13	5	30
Dec. 7–24	990	150	19	7·5	45	990	150	19	7·5	45

The increase at the later date was an extra half ration on account of the greater daily consumption; as before, group I with ten birds did not require food on two occasions.

Both groups increased in weight: group I from 1018 to 1489 or 471 g., group II from 1045 to 1515 or 470 g. per bird. No signs of ill-health were noticed. It was necessary to separate the cocks and hens at the last date. Two main groups were made: group I a white cock and four white hens, group II a black cock and six black hens. The extra cock birds were kept in separate houses. The same diet was continued to the end of the period. The hens then began to lay and another rearrangement of groups was necessary. The white birds (group IV) were compared with group III (see Part II) and the blacks were divided into two groups (I and II) consisting of three hens and a cock, the extra cock being taken from the extra set.

Period 7 *from Jan* 14*th to Feb.* 28*th*, 1921. Numerous experiments on the effect of vitamins on egg laying are conceivable. Since it had been found that

birds are most susceptible to insufficiency of B factor, the effect of extra marmite on egg laying was first tried.

The birds (groups I and II) were given an identical diet except that group II was given double the amount of B factor. To compensate for any extra protein in the marmite the two groups were supplied with the same total quantity, but in the case of group I half was heated in the autoclave at 120° C. for one hour to diminish the B factor. The daily diets were:

Group I						Group II				
Oatmeal g.	Milk cc.	Marmite g.	Heated Marmite g.	Cod liver oil cc.	Lemon juice cc.	Oatmeal g.	Milk cc.	Marmite g.	Cod liver oil cc.	Lemon juice cc.
360	40	6	6	3·5	15	360	40	12	3·5	15

No signs of ill-health were noticed in either group and the average weights remained constant at about 1609 g. for group I and 1638 g. for group II. There was a slight difference in the number of eggs:

	Group I	Group II
Jan. 14–31	10 weighing 45·5 g. on average	4 weighing 47 g. on average
Feb. 1–28	27 „ 51·5 g. „	36 „ 51·5 g. „

There were two double eggs weighing 84 and 73 g.

The egg laying period was too short to yield a definite result, but if any recognition can be given to the actual birds in the groups, those in group I were better and started laying earlier than those in group II, and yet more eggs were laid by group II.

The experiment had to be discontinued at this period.

Conclusions.

Chickens can be reared in confinement on a diet of oatmeal and milk if the three vitamins are all contained in the diet.

Chickens are very susceptible to an insufficiency of B factor in the diet. The amount of B factor required in the diet seems to run parallel to the amount of carbohydrate and fat. It may be represented by 0·5 g. of marmite per 30 g. of oatmeal and per 5 cc. of cod liver oil for 11 birds.

A daily amount of A factor as contained in cod liver oil of 5 cc. and a daily amount of C factor as present in lemon juice of 30 cc. suffice for 11 birds.

The above figures are not to be regarded as minimal figures.

It is probable that the necessary "green" food for fowls supplies extra B factor rather than the expected A and C factors.

It would appear that the leg weakness of chicks is due to insufficiency of B factor in the diet.

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IV. THE REARING OF CHICKENS ON THE INTENSIVE SYSTEM. PART II. THE EFFECT OF "GOOD" PROTEIN.

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Osborne and Mendel [1912, 1914] have shown that the growth of rats depends upon the presence of the amino acid, lysine, in the protein of the diet and that normal or average growth follows if 2 or 3 % of this compound be present in the protein. They have shown further [1916, 1] that proteins vary greatly in their capacity for promoting growth. Lactalbumin was superior to caseinogen and caseinogen to edestin; cereal proteins were not of "good" quality[1]. Buckner, Nollau and Kastle [1915] found that a mixed grain diet of high lysine content produced more rapid growth than one of low lysine content in the case of chicks and this was confirmed by Osborne and Mendel [1916, 2]. Buckner, Peter, Wilkins and Hooper [1919] repeated the experiments with a larger number of birds kept under better conditions at a poultry farm and again noted the effect of a high lysine content. In all these experiments there was a considerable mortality amongst the birds, probably to be accounted for by an insufficient supply of vitamins. In Part I [Plimmer and Rosedale, 1922] it was shown that chickens could be reared in confinement if an adequate quantity of each of the three vitamins were added to the diet. It seemed that the B factor must be present in proportion to the amount of carbohydrate and fat in the diet.

The present experiment was designed partly to confirm the previous one and partly to ascertain the effect of "good" protein upon the rate of growth. A mixture of lactalbumin and caseinogen was chosen; both these proteins contain a high amount of lysine; the main deficiency is that of cystine in caseinogen. Vitamins, additional to those in the commercial foods, were added and oatmeal formed the basis of the diet. The effect of the "good" protein produced the expected result of rapid growth. The cocks reached the average weight of 1828 g. in 122 days; after this time they increased more slowly reaching the average weight of 2002 g. in 144 days; they were really suitable for marketing at the former age. The hens started laying at the age of 139 days.

[1] A summary on "Quality of Protein in Nutrition" is given by Plimmer [1921].

Experimental.

The group (No. III) consisted of nine white Leghorn chicks of the same strains as those in the previous experiment hatched under hens on Aug. 21st, 1920. They were housed for the first three weeks in the foster mother and then in one of the poultry houses in the ground floor room of the Institute, except for a period of 21 days from Sept. 23rd to Oct. 13th, during which time they were kept in a house on the roof of the building. This change made no difference to their rate of growth, which was rapid throughout the experiment.

The diet, except for the first five days, was composed of a mixture of equal parts of caseinogen[1] and secwa[2] or dried whey, mixed with oatmeal. This choice was made so as to give some comparison with the milk used previously. No allowance was made for vitamins in these foods: B factor would be present in the lactalbumin preparation, and both A and B factors in the caseinogen. To ensure an adequate supply they were added in the form of cod liver oil, marmite and lemon or orange juice. The cod liver oil and lemon juice were given, except for a short time at the beginning and again later, in constant daily amounts, but the marmite was increased with the increase of oatmeal required as the birds grew bigger. It was always added in the proportion of 0·25 g. to 30 g. of oatmeal. The food was prepared daily in the same way: the required amounts of oatmeal, caseinogen and secwa were weighed out; marmite was measured from a freshly prepared 25 % solution and mixed with the lemon or orange juice and cod liver oil; some extra water was added and the three ingredients shaken together and well stirred into the food mixture. A wet mash was thus the only food provided; dry food only consisted of the dried surface of the mixture. No extra salts were given and no grit, but on one day the birds were given by mistake some broken pieces of porcelain. The examination of the gizzards of the surplus cocks, after they were killed, revealed the presence of small black stones; they were presumably picked up from the peat moss litter used to cover the floor of the cage.

The quantity of food eaten, protein ratio, average weight and average daily increase are shown in the following table:

	No. of days	Oatmeal g.	Caseinogen g.	Secwa g.	Cod liver oil cc.	Marmite g.	Lemon juice cc.	Protein ratio	Average weight g.	Average daily Increase g.
Aug. 21–25	5	30	0	30	2·5	0·25	15	1 : 6	39–69	6
„ 26–29	4	30	15	15	2·5	0·25	15	1 : 2·5	69–80	3
„ 30–Sept. 3	5	60	30	30	5·0	0·5	30	1 : 2·5	80–111	6
Sept. 4–11	8	90	45	45	7·5	0·75	45	1 : 2·5	111–181	9
„ 12–21	10	120	45	45	7·5	1·0	45	1 : 2·5	181–299	12
„ 22–30	9	180	60	60	7·5	1·5	45	1 : 2·2	299–322	3
Oct. 1–5	6	210	60	60	7·5	1·75	45	1 : 2·5	322–443	20
„ 6–15	10	240	60	60	7·5	2·0	45	1 : 2·4	443–714	27
„ 16–26	11	300	60	60	7·5	2·5	45	1 : 3	714–904	17
„ 27–Nov. 18	23	180	36	36	7·5	1·5	45	1 : 3	904–1233	14
Nov. 19–Dec. 6	18	240	48	48	7·5	2·0	45	1 : 3	1233–1445	12
Dec. 7–26	20	360	72	72	11·0	3·0	67	1 : 3	1445–1668	11
„ 27–Jan. 13	18	360	36	36	7·5	3·0	45	1 : 4	1668–1767	5·5

The diminution in the daily food supply on Oct. 27th was due to the fact that the group consisted of five cocks and four hens and that it was necessary

[1] Caseinogen: moisture 9·9, ash 6·5, fat 0·8, protein 82·7 %.
[2] Secwa: moisture 1·2, ash 9·8, fat 0·3, lactose 74·5, sol. lactalbumin 14·2 %.

to separate off the surplus four cocks (groups III *A* and *B*, below). The figures from this date thus refer to one cock and four hens.

The birds in three weeks reached the weight of the birds in groups I and II [Part I] at the age of six weeks. The cocks in this group began to crow about Oct. 9th at the age of 49 days. The hens began to lay at the age of 139 days on Jan. 7th, 1921. It is recorded that Leghorns have started to lay at 122 days, but these birds were hatched in the spring and had a free existence.

Throughout the period the health of the birds was excellent and they moulted rapidly during October. As might be expected from the work of Palmer and Kempster [1919], the legs and beaks were not pigmented. These investigators showed that the yellow pigmentation depended on the presence of yellow pigments in the food. The food here supplied was almost colourless and the result is a confirmation of their work.

Groups III A and III B. The four cocks after removal from the main group were kept in four separate small cages and were arranged in two groups of two birds as regards food. Both groups were supplied with the same food items as the main group, but with a higher proportion of caseinogen and secwa; this proportion was reduced on Dec. 3rd, so that the protein at first 1 : 1·8 became 1 : 1·3. They were also given more cod liver oil and orange juice per bird than the main group, group III *B* more than III *A*. The marmite was always kept at 0·25 g. per 30 g. of oatmeal. The food consumption, average weight and daily average increase were as follows:

Group III A, each bird.

	No. of days	Oatmeal g.	Caseinogen g.	Secwa g.	Cod liver oil cc.	Marmite g.	Lemon juice cc.	Protein ratio	Average weight g.	Average daily Increase g.
Oct. 27–Dec. 3	39	60	30	30	2·5	0·5	15	1 : 1·8	1053–1730	17
Dec. 4–Jan. 11	39	60	15	30	2·5	0·5	15	1 : 2·9	1730–2202	15

Group III B, each bird.

Oct 27–Dec. 3	39	60	30	30	5·0	0·5	30	1 : 1·8	1015–1587	15
Dec. 4–Jan. 11	39	60	15	30	5·0	0·5	30	1 : 2·9	1587–1804	6

Sometimes the cocks would eat an extra daily allowance during the course of one or more days.

The housing in the smaller cages made no appreciable difference to the daily weight increase, though it was slightly higher than that of the main group. In the first part of the period the greater amount of cod liver oil given to group III *B* had a lessening rather than the augmenting effect which might have been expected. This effect was very marked in the second part. It would support the suggestion made in Part I that the amount of B factor required in the food bears a definite ratio to the consumption of carbohydrate and fat. Cod liver oil was not given for the last few days as the birds were to be killed on Jan. 11th for the purpose of examining the flesh. The flesh of the breast was pale, that of the legs about the colour usual with Leghorns. The flesh was also lacking in flavour, evidently due to the "tasteless" food.

Group III, egg laying period. The hens began to lay on Jan. 7th, 1921; seven eggs weighing 286·5 g. or 40·9 g. per egg were produced up to Jan. 13th.

On Jan. 14th a comparison of egg laying on this diet was made with the same number of hens and a cock (group IV) from the birds of groups I and II. Their diet contained blood meal as protein in exactly the same protein ratio of 1 : 4. Group IV was given less lemon juice, and marmite was omitted from their diet, since it was possible that sufficient of this B vitamin was present in the blood meal. The following tables show the diets, weights etc.:

Group III.

Oatmeal	Caseinogen	Secwa	Cod liver oil	Marmite	Lemon juice	Average weight	Increase
g.	g.	g.	cc.	g.	cc.	g.	g.
360	36	36	7·5	3·0	45	1767–1815	48

Group IV.

Oatmeal	Blood meal	Cod liver oil	Lemon juice	Average weight	Decrease
g.	g.	cc.	cc.	g.	g.
360	87	7·5	45	1537–1456	81

A hen of group III suddenly died on Feb. 4th apparently from choking. No signs of ill-health were noticed beforehand nor could any reason be found at post mortem examination. The other birds were perfectly well throughout.

Group IV did not relish the blood meal; on several occasions it was not necessary to prepare the daily food for them.

Grit was not supplied to either group; the eggs had always a sound shell.

Group III gave	6	eggs	during	January	weighing	253·5 g.	or	42·5 g.	per egg.
„ „ „	17	„		February	„	702·5 g.	„	41·3 g.	„
„ IV „	7	„		January	„	316·5 g.	„	45·2 g.	„
„ „ „	3	„		February	„	134·4 g.	„	44·8 g.	„

The very great difference in the number of eggs from the two groups may have been due to the blood meal in the food of group IV, but from the results recorded in Part I, it is more probably due to absence of B factor. Unfortunately it was not possible to test this supposition, as the experiment had to be discontinued.

Conclusions.

On a diet containing sufficient of each of the three vitamins, chickens can be raised in confinement. This confirms the results of Part I.

Rapid growth results on a diet having an albuminoid ratio of 1 : 3 and containing "good" proteins, caseinogen and lactalbumin. Cock birds began to crow in 49 days and hens to lay in 139 days; the cocks had an average weight of 1853 g. and hens of 1815 g.

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V. DISTRIBUTION OF ENZYMES IN THE ALIMENTARY CANAL OF THE CHICKEN.

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The presence of lactase in the intestines of animals and the non-adaptation of the pancreas and intestine to lactase by feeding with lactose was investigated by Plimmer [1906]. Lactase was always found to be absent from the intestine of chickens. A diet containing lactose had been used by us [1921] in feeding chickens from birth for a period of over three months. Examination of the birds' excreta showed that reducing sugar was absent therefrom, a fact which indicated that the sugar was assimilated. Assimilation of disaccharides is usually preceded by hydrolysis to monosaccharides, which would imply the presence of lactase in the alimentary canal, either in the intestine by adaptation or in some other part. The intestines of the cockerels in this group of birds were therefore examined, after they were killed, for the presence of lactase: it was not found to be present, and the non-adaptation of this organ was verified. If hydrolysis of lactose previous to assimilation occur, it must take place in some other part of the gut. The crop, pancreas and proventriculus were tested and lactase in small amount was detected in the crop. The investigation was then extended to the presence of other enzymes, as no information could be found in the literature about their occurrence in the alimentary canal of birds. The enquiry did not extend to the detection of all known enzymes, but was limited to those concerned in the digestion of the common foodstuffs.

Experimental.

The methods of preparing the enzyme solutions and detecting the presence of enzymes were in general in accordance with those usually adopted; in many cases a longer time of action (up to seven or ten days) was allowed, and in the case of the sucroclastic enzymes, proteins etc. were removed before testing for the reducing sugar formed by their action.

The various parts of the alimentary canal were always taken from chickens killed the same day, or not later than the day previously; on account of the

small size of the crop, proventriculus and pancreas, the organs from four to eight birds were collected and examined together. A single small intestine provided sufficient material, but in most experiments several were combined as the whole series of sucro- or proteo-clastic enzymes were tested for simultaneously. Separate tests were made for lactase. At least two experiments were made with each part, except the caeca.

Preparation of enzyme solutions.

The pancreas, on removal, was cut up into small pieces and ground with sand in a mortar; the ground mass was put into glycerol in which it was kept for several days in the presence of a few cc. of toluene. The solution was then prepared by diluting with rather more than an equal volume of water and filtering from sand, etc.

The other parts of the alimentary canal were cut open and washed with running water to remove the contents. The mucous membrane was scraped off, ground up with sand and water and extracted for 24–48 hours with water in the presence of a little toluene to prevent putrefaction. The aqueous portion was strained off through cloth to remove sand and larger pieces and used for testing for enzymes.

It was not possible to scrape off mucous membrane from the inside of the proventriculus. The organ is glandular, covered with numerous small teats, which, on pressing with a scalpel, emit a yellowish, viscous, distinctly acid secretion. This secretion was the material actually used after grinding with sand and mixing with water. Nothing could be scraped off the gizzard, the interior surface of which resembled parchment.

Detection of enzymes.

(*a*) *Diastase and invertase.* As substrates 100 cc. of 1 % starch solution and 50 cc. of 3 % cane sugar solution were used. Two portions were measured out with a pipette in separate flasks; a known volume of enzyme solution was added to one, and the same volume of boiled enzyme solution, after cooling, to the other; 2 or 3 cc. of toluene were added to each, the flasks corked and put into an incubator at 37° for one or more days. A test for starch by the iodine reaction was made from time to time with a drop removed from the mixture. At the end of the reaction time, the mixtures were washed into a 250 cc. measuring flask, a slight excess of colloidal ferric hydroxide added, any excess of the latter removed by a few crystals of magnesium sulphate, the volumes made up to the mark, the solutions filtered and reducing sugar tested for by the complete reduction of 10 cc. of Fehling's solution. The control solutions containing boiled enzyme did not reduce, or only gave a slight reduction due to sugar present in the extract.

(*b*) *Lactase.* The detection of lactase was carried out in a similar way to that of diastase and invertase, using 50 cc. of 4 % lactose solution as substrate. The enzyme and control mixtures were put directly into 250 cc. measuring flasks

and made up to volume after clearing with colloidal ferric hydroxide and magnesium sulphate. The reducing sugar was estimated by the reduction of 10 cc. of Fehling's solution. The observed difference in reading indicated whether hydrolysis had or had not occurred. No difference in reading was observed in the case of the intestine or proventriculus, but a small though distinct difference was always noticed in the case of the crop extract; it varied from 0·2 to 0·5 cc. in a total of 10 or 10·1 cc. This slight difference indicated an hydrolysis of 10–20 % of the lactose.

(*c*) *Lipase.* This enzyme was not looked for except in the case of the pancreas. Two exactly equal portions of oil in separate test tubes were made just alkaline to phenolphthalein with 0·1 *N* caustic soda. Enzyme and boiled enzyme solution were added. On keeping at 37° and occasionally shaking, the pink colour of the tube containing enzyme solution disappeared and it was restored by adding a few drops of the soda. This could be repeated several times and altogether from 1–2 cc. of alkali were added; the control tube did not change colour.

(*d*) *Proteoclastic enzymes.* Proteoclastic enzymes were detected by their action on Congo-red fibrin in neutral, acid and alkaline media. In the first case, a definite volume of enzyme solution and the same volume of boiled enzyme solution were put into separate flasks; in the other cases the same volumes of enzyme and boiled enzyme solutions were mixed with an equal volume of 0·2 *N* hydrochloric acid or 0·2 *N* sodium carbonate solution in separate flasks; 1 g. of Congo-red fibrin and 2 cc. of toluene were added to each and the several flasks were put in an incubator at 37° for one to seven days. Solution of Congo-red fibrin, which, in the case of hydrolysis, generally occurred in one or two days, was taken as indication of the presence of proteoclastic enzyme; solution did not occur in those flasks with boiled enzyme solution. No investigation was made of the products of the hydrolytic action.

Results.

The presence or absence of enzymes in the various parts of the alimentary canal is most easily seen from the following table:

	Crop	Proventriculus	Pancreas	Intestine whole	Duodenum	Ileum	Caeca
Invertase	0	0	.	+	.	.	0
Diastase	+	0	+	+	.	.	+
Lactase	+	0	.	0	.	.	.
Lipase	.	.	+	.	.	.	.
Proteoclastic in neutral	0	0	+slight	0	0	0	0
„ acid	+slight	+	+less rapid	+	+	+	0
„ alkaline media	0	0	+rapid	+slight	+	+slight	0

The distribution of the sucroclastic enzymes corresponds in most particulars with that in the animal; most animals have invertase in the intestine, lactase is present in some, absent in others: diastase and lipase are generally

present in the pancreas of animals. The proteoclastic enzymes show a difference: the animal has trypsin acting in alkaline media; the chicken in both alkaline and acid media. The intestine of the chicken has an enzyme acting most rapidly in acid medium, less rapidly in alkali. The proteoclastic enzyme of the proventriculus acts only in acid medium; the organ corresponds to the stomach of animals. The caeca, as expected, had no enzyme of this group, but contained diastase.

We wish to thank Prof. J. A. MacWilliam, F.R.S., for kindly allowing us to carry out these experiments in his laboratory.

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VI. THE AMINO-ACIDS OF FLESH.

THE DI-AMINO-ACID CONTENT OF RABBIT, CHICKEN, OX, HORSE, SHEEP AND PIG MUSCLE.

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(*Received December 20th, 1921.*)

A long series of food analyses has recently been made by Plimmer [1921, 1], who points out that by the ordinary routine method of analysis, in which the amount of protein is estimated by multiplying the nitrogen content by 6·25, no discrimination is made between the flesh of different animals. The protein of one animal is regarded as being the same as that of another. The work of Emil Fischer and Kossel and their pupils has definitely proved that the various proteins differ very widely in their composition as regards the amino-acids, and this difference is emphasised by the experiments on the food value of the individual amino-acids by Hopkins in conjunction with Willcock and Ackroyd, by Osborne and Mendel and other American investigators[1]. These chemical and biological differences are sufficient evidence that quality of protein in nutrition must be taken into consideration.

Complete analyses of the protein of the muscle of the ox, chicken, halibut and scallop have been made by Osborne and Heyl [1908] and Osborne and Jones [1909], and Drummond [1916] has made some analyses of muscular tissue by Van Slyke's method. Both the more complete analyses by Osborne and co-workers and those by Drummond do not show any marked difference in the amino-acid content of the various muscle proteins. The flesh of various animals shows such distinct appearances, different both to the eye and palate, that it seems probable that greater differences may exist, and that there may be smaller differences in the flesh from various parts of the same animal's carcase, such as back and leg. Some further amino-acid analyses have therefore been made.

The methods of protein analysis are far from perfect: Fischer's ester method for the mono-amino-acids, as he pointed out, is not quantitative: Kossel and Patten's method for the di-amino-acids, in spite of the numerous manipulations, is generally considered to be fairly accurate, but it has been largely superseded by Van Slyke's method which gives higher values for these amino-acids. Van Slyke's method also possesses the advantage of requiring only small amounts of protein and is more rapidly carried out. This method of protein analysis has been used in these experiments, since it was chiefly

[1] See summary by Plimmer [1921, 2].

desired to compare the muscle protein of several animals with a view to more complete data at a later time. A comparison of these results with those by Kossel's method has been made in a few cases. The results indicate that differences exist in the amino-acid content of the various muscle proteins. Duplicate analyses were always carried out; frequently these analyses were not so concordant as was expected. This inconsistency of the results was under investigation by Plimmer [1916] who tested the arginine determination; other details of the method are now being studied.

Experimental.

In the case of the smaller animals (rabbit, chicken) opportunity was taken of comparing the flesh of different parts of the body of the same animal. In other cases the flesh was taken from the thigh. The mode of operation was the same throughout. The flesh (about 350 g.) was freed from inside fat, minced and put into about 2 litres of boiling water containing 0·1 % acetic acid and heated for about ten minutes so as to coagulate the protein and remove the extractives. The liquid was poured off and the coagulated protein squeezed dry in a cloth. This procedure was repeated twice. The coagulated protein (about 200 g.) was then digested with 1 g. pepsin in 2 litres of 0·1*N* HCl, so as to separate nucleins, indigestible matter, etc. After digestion, which usually occupied about ten days at 37° the liquid was filtered off and the total nitrogen estimated. A portion containing about 6 g. of protein was then hydrolysed by boiling with hydrochloric acid added to the liquid so as to make a concentration of 20 %. The hydrolysis was carried on for 36 hours. The hydrolysed solution was evaporated to dryness *in vacuo*, made up to 250 cc. and two samples of 100 cc. were analysed by Van Slyke's method. This was performed as described except for the arginine estimation which was effected by Plimmer's modification [1916]. In the earlier experiments it was impossible to make determinations of amide N owing to the facilities for vacuum distillation not being adequate. The analyses were made in duplicate and the percentage has been calculated from the average.

Table I. *Nitrogen percentages.*

			Di-amino-acids						Mono-amino-acids			
	Amide	Humin N	Total N	Amino N	Non-amino N	Argi-nine N	Histi-dine N	Lysine N	Total N	Amino N	Non-amino N	Total N of hydro-lysed solution
Rabbit, back	—	—	45·7	21·5	24·0	15	19	11·5	49	—	—	94·7
„ fore limb	—	—	44·1	17·9	27·7	8·8	30·9	5·5	50·7	—	—	94·8
„ hind limb	—	—	44·8	18·4	26·6	13	25	5·8	56·3	—	—	101·1
Chicken, breast	6·9	3	27	9	18	10	13	2	61·1	49·7	11·4	98·0
„ legs	5·5	1·3	25·5	15	10·5	8	7	11	68·5	66·6	1·9	100·8
Beef	6·3	0·5	28·5	15	13·5	13·3	5	11·2	55	26·8	28·2	90·3
Horse	2·9	0·9	37·1	18·8	18·3	14·9	10·5	11·6	70	58	11·9	110·9
Mutton	6·5	0·5	38·3	22·3	15·6	15	18	4·3	54	52	2	99·3
Pork	6·4	1·2	28·2	13·3	15	14	7	7	57	53	4	92·8

Relatively little difference can be observed from the figures for the different meats. The amide N is almost similar, in each case averaging about 6·0 % of the total N.

Table II. *Percentages of amino-acids. Giving the amount of amino-acids in 100 g. of protein.*

	Arginine	Histidine	Lysine	Total di-amino N
Rabbit, back	8	10	13	31
„ fore limb	5	19	5	29
„ hind limb	7	15	5	27
Chicken, breast	6	8	1	15
„ legs	4	4	10	18
Beef	7	3	10	20
Horse	7	6	9	22
Mutton	7	11	4	22
Pork	7	4	6	17

Humin N shows a difference. It is, if anything, higher in the white meats, *e.g.* breast of chicken 3 %, legs 1·3 %, pork 1·2 %, than in the red meats, where the average is 0·5 %, except in the horse, where 0·9 % was found. The explanation of this slightly higher value may be that the animal was not properly bled on slaughter.

Lysine figures are, with the exception of mutton, higher for the red meats, averaging about 11 %, while, of the white meats, rabbit limbs show only 5·5 %, chicken breast 2 % and pork 7 %.

Gortner and Holm [1920, 1], working with mixtures of pure amino-acids, have shown that tryptophan, and in the presence of aldehyde also tyrosine, and their analogues are the only known amino-acids which go to form humin. There is therefore no connection between the humin content and the lysine content of the meats; this is exemplified especially in the chicken, where the humin is high, and the lysine is low in the breast; and humin is low and lysine high in the legs. It may perhaps be mentioned that in the preparation of the di-amino acids by the method of Kossel and Patten a distinct yellowish colouring adheres to the lysine portion.

At the same time too much reliance must not be placed on the humin as an estimation of tryptophan and tyrosine. Gortner and Holm [1920, 2] and Thomas [1921] have shown that tyrosine and tryptophan which go to form humin are not necessarily the only substances giving a reaction with the phenol reagent of Folin and Denis. Estimations of substances giving the blue colour with this reagent were made during the progress of this work, both before the removal of the humin and afterwards. In the case of chicken breast, a white meat, the readings before removal of the humin represented 4 % "tyrosine" whereas after its removal the readings represented 3·5 %. In the case of beef however—a red meat—the difference was greater, the former reading being 3·5 % and the latter 2·1 %, yet the humin N was much lower in the case of beef.

The arginine figures are more constant at about 14 or 15 % except in rabbit fore limb and chicken legs where the average is 8 %.

The histidine figures are less satisfactory, and exhibit perhaps a weak point in the method. In this connection it is of interest to point out that in the cases of abnormally high histidine the figures for the non-amino N are lower than normal and *vice versa*, *e.g.* beef 5 % histidine, 28 % non-amino N,

mutton 18 % histidine, 2 % non-amino N, while in other cases this observation cannot be made. This may be due, either to incomplete precipitation of the histidine by the phosphotungstic acid, or to washing. Work in this connection is in progress.

It is not possible to draw any conclusions from the figures of the mono-amino fraction, which account for about 55 to 60 % of the total N.

The average percentage of the di-amino N is 35.

Comparison with former work on the hydrolysis of meat is difficult, because, with the exception of Drummond [1916] on chicken meat, the other figures relate to the method of Kossel, which generally gives lower results than the Van Slyke method.

The above figures for chicken breast agree in the main with those of Drummond, his total hexone bases N 27·26 being the same as that above. The arginine figures are within 1 % and he records having used the same modification of that process as mentioned above. The figures for histidine and lysine are discordant, Drummond finding 8·45 and 9·81 respectively, while the total N of the mono-amino fraction is 4 % higher than that found by Drummond.

In order to compare the figures of Osborne and co-workers with the above it is necessary to refer to the percentages not of total N but of actual arginine, histidine and lysine. The figures for arginine are generally constant within 1 %, those for histidine are higher than Osborne's, while the lysine figures, owing to the calculation in Van Slyke's method, are dependent on the histidine values. Apart from the arginine values, only the beef of the present sets has given results comparable with those of Osborne, who found 7·5 % arginine, 1·8 % histidine and 7·6 % lysine against 6·8 % arginine, 2·6 % histidine and 9·6 % lysine in this experiment.

Summary.

1. Determinations have been made of the di-amino-acids of the protein of the flesh muscle of rabbit, chicken, ox, horse, sheep, pig by Van Slyke's method.

2. The red meats show a higher lysine content than the white meats.

I wish to take this opportunity of expressing my gratitude to Dr Plimmer, who suggested this work, for his kindness and guidance throughout the time I was under him, and also to Professor J. A. MacWilliam, F.R.S., for so kindly placing his laboratory at my disposal.

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VII. XYLENOL BLUE

AND ITS PROPOSED USE AS A NEW AND IMPROVED INDICATOR IN CHEMICAL AND BIOCHEMICAL WORK.

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(*Received December 24th, 1921.*)

During the course of some studies on the most important of the sulphonephthaleins synthesised by W. M. Clark and Lubs [1917], the writer was led to synthesise a new sulphonephthalein which has practically the same properties as thymol blue but with the important advantage of being twice as intense and therefore more economical.

Lubs and Acree [1917] in their earlier quests for evidence on the quinonephenolate theory, now practically firmly established, noticed various relationships between the chemical constitution of the sulphonephthaleins and the corresponding working ranges of utility. Thus the introduction of negative (bromine) groups shifts the working ranges to a more acid region, *e.g.* cresol red with a working range from P_H 7·2 to P_H 8·8 on bromination yields brom cresol purple with a working range from P_H 5·2 to P_H 6·8, a shift of two units for either limit. The same shift occurs on brominating thymol blue to produce brom thymol blue. On brominating phenol red to produce brom phenol blue, a tetra-brominated instead of a dibrominated sulphonephthalein results, and the shift is four units for either limit of the working range.

Whilst then it would appear that negative group-substituents induce fairly regular changes of P_H ranges, another interesting aspect of the matter is presented by the shifting of the ranges towards the more alkaline region, by the introduction into the phenol residue of phenol red of positive groups like methyl and *iso*-propyl as in cresol red and thymol blue, which is a 1-methyl-4-*iso*-propyl-3-phenolsulphonephthalein. Here, however, the well defined acid range of thymol blue complicates matters.

It occurred to me to compare the case of diethyl red and dipropyl red. These are azo indicators and homologues of the well-known methyl red, or more strictly dimethyl red, dimethylaminoazobenzene-*o*-carboxylic acid. The two homologues show the same P_H range, viz. P_H 4·5–6·5, in spite of the increased positive character of the propyl groups. The following show similar anomalous behaviour.

Table I.

1. Methyl Orange: Sodium *p*-dimethylaminoazobenzenesulphonate 2. Mono-ethyl Orange: Sodium *p*-monoethylaminoazobenzenesulphonate	P_H 3·1–4·4
3. Sörensen's: α-naphthylaminoazobenzene α-naphthylaminoazo-*p*-toluene	P_H 3·7–5·0
4. Sodium *p*-benzylanilineazobenzenesulphonate 5. Sodium *p*-aminoazobenzenesulphonate	P_H 1·9–3·3

One would suspect therefore the possibility of a similar anomalous unshifted P_H range when the thymol residue in thymol blue is replaced by *p*-xylenol; the difference here is in the replacement of the *iso*-propyl group in thymol by another methyl radical.

In view of this, it occurred to me, that owing to the smaller molecular weight, the resulting new sulphonephthalein should be used like phenol and cresol reds in 0·02 % solution to produce the same intensity as 0·04 % thymol blue or in strength between the two percentages. We should then have a more symmetrical list in which only the brominated dyes are used in the higher concentration of 0·04 % (or 1·2 % concentrated), alcoholic or aqueous monosodium salt, whenever phenol and cresol reds are chosen in half these strengths. These relative concentrations among the sulphonephthaleins have been recommended largely on the basis of equality of *depth* of colour, as far as can be judged by the eye with the reds and blues, though the reds and blues do not appear of the same *quality*, owing mainly to difference in degree of dichroism.

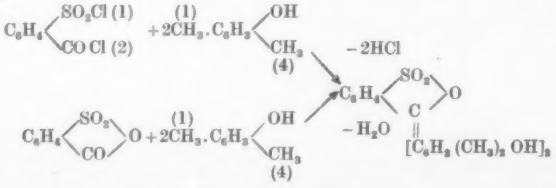
By condensing the chloride or anhydride of *o*-sulphobenzoic acid with *p*-xylenol, a product was ultimately obtained having the same double P_H ranges as thymol blue and similar colour changes.

Experimental.

The method of preparing 1·4-dimethyl-5-hydroxybenzenesulphonephthalein or xylenol blue is as follows [Chemicals & Bye-products, Ltd., and A. Cohen, 1921]:

A mixture of ten parts of *o*-sulphobenzoic dichloride, or of the acid anhydride; ten parts of fused zinc chloride; and fifteen parts *p*-xylenol, M.P. 74·5°, B.P. 211·5°, is heated in a brine bath for six hours. The melt is heated with forty parts water till disintegrated. It is then filtered hot, washed with hot water, and afterwards with a little alcohol. Next, it is dissolved in excess caustic soda and precipitated with hydrochloric acid whilst being stirred. The mixture is filtered and crystallised from alcohol in the form of a brown solid.

The reactions are:



Samples of the new indicator were titrated against 0·01 *N* NaOH using a micro-burette up to 5 cc. and reading to 0·01 cc.

Sample 1. 0·0205 g. in 150 cc. water (yellowish orange) required 4·90 cc. instead of the theoretical 5·00 cc. or 98 % molecular equivalent alkali.

Two other samples gave identical results.

Carbon dioxide must of course be rigidly excluded in such titrations involving high dilutions.

As in the cases of phenol and cresol reds, a 0·6 % aqueous solution of the monosodium salt can be made by gently boiling, not merely warming, for 3 minutes, 0·6 g. of the solid powdered dye in about 80 cc. water containing 1·47 cc. of normal alkali, and then diluting when cold to 100 cc.

If preferred, a strong alcoholic solution can be made by boiling 0·2 g. of the solid in 100 cc. absolute alcohol.

A 0·02 % solution was prepared by a thirty-fold dilution of the 0·6 % aqueous monosodium salt solution, and added to cordite tubes (or test tubes of hard white glass, unflanged and of uniform diameter) each containing 10 cc. of a standard buffer solution. Using a concentration of 0·05 cc. or 1 drop indicator per cc. buffer solution, the double series P_H 1·2–2·8 and P_H 8·0–9·6 (each in consecutive steps of 0·2 P_H) with xylenol blue, were similar to those with thymol blue added as a 0·04 % solution in the same concentration of 0·05 cc. per cc. The tints were, in fact, rather more distinct with xylenol blue in the alkaline range, appearing a purer blue in the daylight. Further, unlike dipropyl red in the anomalous case mentioned above, xylenol blue does not tend to precipitate out from buffer solutions even after three months' standing. On the contrary, xylenol blue stands in this respect to thymol blue, as the preferred diethyl red stands to dipropyl red.

Like thymol blue, it can be used for the estimation of the P_H of gastric juice and ammoniacal fluids; and in the differential acidimetric and alkalimetric titrations as described by A. B. Clark and Lubs [1918].

In Table II some results are given, obtained with mixtures of hydrochloric and benzoic acids. A 5 cc. micro-burette graduated in hundredths of a cubic centimetre was used, and I have found it convenient to use a special easily constructed titration flask in which a cordite tube is fused horizontally to the side of the flask to facilitate colorimetric comparison with the standard coloured benzoic acid. The cordite tube attachment also serves to stir the liquid when the flask is tilted. Such a flask can be heated without any cracks developing in the joint, provided the heating is done at the side remote from the joint. I wish here to thank one of my colleagues Mr G. H. Wallis for his kindness in constructing this flask.

0·5 cc. of 0·02 % aqueous xylenol blue was added to 50 cc. of an unknown benzoic acid solution, and 10 cc. of this standard kept in a cordite tube. The 0·5 cc. indicator is arbitrary and others may find it better to use more or less, but the less indicator is used the better; the differentiating power of the eye being an inadequately known function of subjective colour differences.

The mixtures titrated had, of course, the same concentration of indicator as the standard.

Table II. *Titration of mixtures of hydrochloric and benzoic acids.*

	Benzoic acid cc. used	1 *N* HCl cc. used	0·993 *N* NaOH to standard	0·993 *N* NaOH to blue	*N* HCl found cc.	G. of benzoic acid per cc. found
1	10	nil	nil	0·39	nil	0·0047
2	10	3·03	3·06	3·46	3·04	0·0049
3	25	4·04	4·05	5·00	4·02	0·0046

Experiments are in progress on the conditions for brominating xylenol blue.

Summary.

p-Xylenolsulphonephthalein or xylenol blue, a new indicator having two working ranges of utility from P_H 1·2 (red) to 2·8 (yellow) and from P_H 8·0 (yellow) to 9·6 (blue), can be successfully employed in all work for which thymol blue has heretofore been recommended.

The fact, that *p*-xylenol is easily prepared from diazotised *p*-xylidene and that only half as much xylenol blue as thymol blue is required, should render its use eminently preferable.

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VIII. THE EXAMINATION OF SOME INDIAN FOODSTUFFS FOR THEIR VITAMIN CONTENT.

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(*Received January 3rd, 1922.*)

THE subject of this paper was taken up at the suggestion of Dr J. C. Drummond, with a view to ascertaining whether the principal foodstuffs consumed by the mass of people in Bengal contain adequate amounts of different vitamins or not.

Among the previous workers in this field mention should be made of Chick and her collaborators [1917, 1919], who tested certain cereals and flours and samples of dried fruits for the presence of vitamin *B*, etc. and Greig [1917, 1918], who examined the anti-scorbutic value of Indian grains and lentils. Shorten and Roy [1921] also examined some vegetables for vitamins *B* and *C*. Chick had found that pure white flour is deficient in vitamin *B*. Chick, Greig and Shorten found that vegetables and lentils under certain conditions contained good amounts of anti-scorbutic vitamin.

The problem of supplying foodstuffs which should contain liberal amounts of different vitamins is very important in Bengal. Beri-beri occurs to some extent, and scurvy and rickets are encountered but are not widespread. From this but more from the low resistance of the people to various infectious diseases, and the presence of certain forms of eye-diseases, it may be suggested that the mass of the industrial classes may be living at or below the danger level of vitamin deficiency.

The table given below—quoted from MacCay [1912]—shows typical diets ordinarily in use among the different classes in Bengal.

Table I.

	Cultivators ozs.	Middle class (not above indigence) ozs.	Middle class (above indigence) ozs.	Well-to-do ozs.
Rice	20	16	6	4
Dal	1	1	1	½
Vegetables	4	4	4	4
Oil	1	—	1	1
Fish	1	1	2	4
Wheat flour	—	—	6	6
Milk	—	—	8	16
Butter	—	—	1	4
Calories (about)	2390	2310	2350 (Meat and eggs also added)	2950 – 3450 (Meat and eggs are taken, also sweets freely)

From this table it will be seen that so far as calorific values, etc. are concerned, the diets of the people are fairly in compliance with accepted standards. Low resistance to disease, therefore, is probably due to vitamin deficiency—vitamin *A* specially—although MacCay is of opinion that this low resistance is due to want of sufficient protein in food.

The above table may be regarded as roughly accurate, but MacCay omits to emphasise that all classes of the people eat large quantities of fruit during the spring and summer months; also that the amount of lentils consumed by the poorer classes is much higher than that in the table.

The principal "fatty foods" consumed are "ghee," various preparations of butter, mustard oil, and, to a limited extent, coconut oil. These are mainly used in cooking.

Experimental.

This enquiry has been confined to an examination of different samples of "ghee," mustard oil and coconut oil for the presence of vitamin *A*; "dals," *i.e.* lentils, and some samples of Indian flours, for vitamin *B*.

"Ghee" is made either from cows' milk or buffalo milk. As a rule, the raw milk is first warmed over a low flame for about half-an-hour, and then cooled and inoculated with sour milk. After about 12 hours, when curdling is complete, water is removed and the curd churned until the fats separate out. The fat is removed, clarified, and then cooled into a semi-solid mass of buttery consistency. In some cases this product is allowed to turn rancid and then heated and rendered.

Fresh "ghee" remains good for months if well stored in the dark.

The following table gives the analysis of different "ghees." The results are similar to those quoted by Bolton and Revis [1910].

Table II.

	Reich.-M. value	Sapon. V.	Iodine N.	Free fatty acids
Pure cows' "ghee" (yellow)	28·7	230	31	2·2
Pure cows' "ghee" (remelted) eight months old sample (yellow)	26	229	29	2·6
Pure buffalo "ghee" (white)	38·8	224	27·2	—
Adulterated "ghee" (white)	14·4	213	29·3	—

"Ghee" is frequently adulterated with lard, mutton fat, coconut oil, and almond oil.

Rats about one month old, which were over 50 g. in weight, were selected and put on a diet deficient in vitamin *A* [Drummond and Coward, 1920]. Generally it took about three weeks for the rats to become steady in weight and fit for the feeding experiment. Then each rat was given 0·2 g. of "ghee" every morning in addition to its usual basal diet.

In the majority of cases the rats made steady increase in weight for about ten days (sometimes varying from 10 to 21 days) when they again became

stationary. The amount of "ghee" was then raised to 0·4 g. a day, when the rats again resumed growth.

It is to be noted that the remelted sample did not induce growth, but some of the adulterated samples were fairly active when given as 5 % in basal diet.

It has been shown by Drummond, Coward and Watson [1921] that 0·2–0·4 g. per day of average samples of butter is sufficient to maintain growth for rats.

From the examination of the table of weighings it will be seen that both pure cows' "ghee" and buffalo "ghee" may be as good as European butters.

Table III.

(Showing the growth of rats on *A*-deficient diet and 0·2 g. of pure "ghee." The weighings of the rats during the preparatory period are omitted.) g.

	931, male	835, male
First weighing	91	58
After 10 days	102	74
„ 15 „	104	83
„ 30 „	124	100
„ 40 „	138	110

Table IV.

(Rats on *A*-deficient diet + 0·2 g. of pure "ghee." Sample 2.) g.

	832, male	834, male	940, male
First weighing	80	89	56
After 10 days	85	111	77
„ 15 „	90	120	76*
„ 30 „	120	150	105
„ 40 „	129	162	112

* Amount of "ghee" doubled

Table V.

(Rats on remelted cows' "ghee.") g.

	833, male	836, female	828, male
First weighing	90	55	80
After 10 days	94	60	66
„ 15 „	92	52	60
„ 30 „	95	—	—
„ 40 „			

Table VI.

(Rats on pure buffalo "ghee.") g.

	933, male	1176, male	932, female
First weighing	104	76	95
After 10 days	120	105	109
„ 15 „	—	—	—
„ 30 „	136	150	136
„ 40 „	151	—	148

Table VII.

(Rats on adulterated "ghee." Sample I.) g.

	1510, female	1177, male	1176, male
First weighing	72	65	68
After 10 days	78	68	73
„ 30 „	75	72	72
„ 40 „	80	76	74

Table VIII.

(Rats on adulterated "ghee." Sample II, given as 5 % in basal diet.) g.

	976, female	1300, male
First weighing	80	60
After 10 days	—	—
„ 30 „	110	95
„ 40 „	125	109

Table IX.

(Rats on coconut oil and *A*-deficient diet. The oil was given in the basal diet 10–20 %.) g.

	1457, male	1456, male	1463, female
First weighing	82	64	76
After 10 days	95	69	94
„ 20 „	118	77	105
„ 30 „	130	91	102

Table X.

(Rats on mustard oil: 10–20 % in the basal diet.) g.

	1508, male	1483, female	1511, male
First weighing	77	57	64
After 10 days	84	62	76
„ 20 „	88	64	85
„ 30 „	98	72	92

Some of the adulterated samples gave good results, but as this depends on the adulterants used, the different samples gave widely varying results.

Daniels and Loughlin [1920] and Drummond, Golding, Zilva and Coward [1920] have shown that lard may contain a certain amount of vitamin *A*, the latter authors having shown that this occurs when the pigs have been grass-fed, and it should be borne in mind that lard is a most widely used adulterant in "ghees."

Contrary to the results of previous workers [cf. Jansen, 1920], the samples of coconut oil examined by me did produce fair growth in rats. It should be mentioned here that coconut oil was not administered in the same way as "ghees," but was incorporated in the basal diet in 10 % proportion and replaced hardened fat.

The results obtained with pure mustard oil are also interesting. The oil was administered in large quantities (10–20 % in diet) to the rats, and in some cases actual growth was obtained contrary to the results of previous workers. Both mustard oil and coconut oil were those prepared in India and most probably not so refined as in Europe or America.

The buffalo "ghee" is pure white, and the presence of the growth-promoting vitamin *A* in it confirms Drummond and Coward's work on vitamin *A* and colour [1920].

That renovation under certain conditions does not affect the vitamin content is also in accord with the results found by Drummond, Coward and Watson [1921].

Examination of different samples of lentils.

The procedure for examining the samples of lentils was similar to that adopted in the case of "ghees," except that in these experiments the rats had been previously fed on diet deficient in water-soluble *B*. Each rat, after it had begun to lose weight on this diet (*B*-deficient diet), was given 1 g. of the lentil to be tested, daily.

The *B*-deficient diet was similar to the Drummond-Coward diet (*A*-deficient) except that no yeast extract was given and that butter replaced hardened fat.

The weighed amount of lentil (1 g.) was kneaded with a small portion of the diet and given in the morning before the rat had the main bulk of its food.

The different lentils thus examined included:

1. "But" (yellow variety)—*Cicer arientinum* Linn.
2. "Sona Moong" (golden-yellow in colour)—*Phaseolus Mungo* Linn.
3. "Arhar" (small size, yellow-ochre variety)—*Cejanus Indicus* Spreng.
4. "Lal Moong" (red variety)—*Phaseolus radiatus* Linn.
5. "Mash Kalai" (greenish variety)—*Phaseolus Mungo* Linn.
6. "Bara Lal But" (red variety)—*Cicer arientinum* Linn.

It should be mentioned here that as far back as 1900 Grijns [1901] working in Java found "Katjang Hidjoe"—a variety of *Phaseolus radiatus*—a good cure for both human and avian beri-beri. Hulsoff [1917] also worked with the

anti-neuritic property of Katjong (*Phaseolus radiatus*). A similar result has also been found by Pol [1917].

Table XI. *Composition of the various "Dals" (lentils).*

Name	H_2O %	Ether extract %	Protein %	Soluble carbo-hydrates %	Woody fibre %
5. *Phaseolus Mungo* Linn.	8·5	0·9	19·5	59·3	4·5
2. „ „ Yellow	10·99	0·83	20·5	59·0	4·8
4. *Phaseolus radiatus.* Red	10·0	0·93	22·9	58·9	3·38
1. *Cicer arientinum.* Yellow	11·4	4·9	18·6	56·3	6·1
6. „ „ Red	8·6	5·3	15·5	60·1	7·2
3. *Cejanus Indicus* Spreng.	14·3	1·9	15·8	57·2	5·8

Examination of the tables showing the growth of the rats on the lentils, shows that all the above varieties of lentils contain appreciable amounts of vitamin *B*.

Table XII.

Rats on *B*-deficient diet (1 g. of lentil a day). (*Phaseolus Mungo*—greenish variety.) g.

	1262, female	1267 *a*, female	1267, female
First weighing	90	102	75
After 10 days	117	122	95
„ 20 „	133	138	113

Table XIII.

Rats on *Phaseolus Mungo*—golden-yellow variety. g.

	1282, male	1284, male	1285, male
First weighing	92	100	80
After 10 days	109	130	100
„ 20 „	122	151	115

Table XIV.

Rats on *Phaseolus radiatus.* g.

	1258, male	1283, male	1259, female
First weighing	72	100	63
After 10 days	82	140	85
„ 20 „	86	162	104

Table XV.

Rats on *Cejanus Indicus.* Spreng. g.

	1260, female	1261, male
First weighing	86	95
After 10 days	100	131
„ 20 „	115	146

Table XVI.

Rats on *Cicer arientinum.* Yellow variety. g.

	1264 male	1265 female	1266 female
First weighing	76	76	90
After 10 days	68	94	115
„ 20 „	78	110	128

Table XVII.

Rats on *Cicer arientinum.* Red variety. g.

	1274 male	1277 male	1276 male	1275 male
First weighing	82	96	80	95
After 10 days	98	130	101	115
„ 20 „	105	146	112	127

Table XVIII.

Rats on *B*-deficient diet and 1 g. of pure unbleached Indian flour. g.

	1278, male	1279, female
First weighing	106	88
After 10 days	120	115
„ 20 „	135	120

Table XIX.

Rats on crude "Attah." g.

	1266*a*, female	1268*a*, female	1269*a*, male
First weighing	83	75	65
After 10 days	101	105	98
„ 20 „	111	117	117

Table XX.

Rats on bleached Indian flour. g.

	1292*a*, male	1291*a*, female	1293*a*, female
First weighing	81	69	76
After 10 days	80	65	73
„ 20 „	79	Dead	Dead

Examination of different samples of Indian flour.

Here the samples examined were only those that are widely used in Bengal. They were:

1. Crude "attah." ("Attah" is a mixture of various flours, *e.g.* wheat, pea and maize, etc., and contains the husks of the cereals in it.)

2. Pure Indian flour. This flour has a finer texture than the "attah." It is machine-ground, but it contains the ground-up husks of the cereals. This flour is not bleached.

3. White flour. This is machine-ground like the pure Indian flour, but it does not contain the husks, and moreover it is bleached perfectly white. Sometimes chlorine is added in the bleaching process.

1 g. of each of the above samples was given daily to the different sets of rats, in the same way as in the case of lentils.

The composition of the different flours is given below.

Table XXI.

	H_2O	Ether extract	Carbo-hydrate	Protein $N \times 5{\cdot}7$	Crude fibre
1. Crude "Attah"	14·6	2·9	67·1	11·5	3·9
2. Pure unbleached flour	15	2	71·2	11	0·8
3. Bleached white	12	2	71·3	13·5	0·6

The results found are in accord with those of Chick as well as with the facts announced by Col. Hehir [1919, 1, 2] in the Mesopotamia Commission Report on the presence of deficiency diseases in the besieged garrison in Kut. Willcox [1917] has also discussed "attah" as a protective against beri-beri.

Johns and Finks [1920] found that a mixture of pea-flour and wheat-flour produces better growth in rats than simple wheat-flour, and these facts are also corroborated since "attah" is found to be better in promoting growth than pure (unbleached) flour. From the weighings of the rats, fed on the different samples of the flours, it will be seen that with the exception of bleached flour they contain appreciable amounts of vitamin *B*.

Conclusion and Summary.

Samples of pure Indian "ghee" proved to be as good a source of vitamin *A* as pure butter.

Samples of remelted "ghee" ("makkum galana") failed to restore growth in rats whose weights had become stationary on a shortage of fat-soluble *A*.

Two samples of "ghee" whose history is unknown failed to promote growth.

Some samples of adulterated "ghee" only gave growth in rats when administered in large quantities (*e.g.* 5 % of the basal diet).

Certain edible vegetable oils, *e.g.* ("narikel") coconut oil, and pure mustard-seed oil were examined, and these were found to give growth when administered in 10–20 % in the basal diet.

Various samples of lentils were examined and these showed good content of vitamin *B*.

Bleached (pure white) Indian flour is found to be deficient in vitamin *B*, but both crude "attah" and unbleached Indian flour have considerable quantities of water-soluble vitamin *B*.

In conclusion I take the opportunity of expressing my thanks to Dr J. C. Drummond and Miss Katharine H. Coward, for kind help and advice.

My thanks are also due to Mr Suvaga for kindly giving me some samples of remelted "ghee," and to my brother, Mr B. N. Ghose, for various samples of lentils, flours, oils and "ghees."

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IX. CONDITIONS OF INACTIVATION OF THE ACCESSORY FOOD FACTORS.

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(*Received January 4th, 1922.*)

In some preliminary communications [Zilva, 1920, 1921] attention was drawn to the fact that the fat-soluble and the anti-scorbutic factors were inactivated on exposure to ozone in the case of the former and ozone and air in the case of the latter. It was further pointed out that the anti-scorbutic factor could withstand the temperature of 100° for two hours when air was excluded. These observations have found confirmation in the results obtained independently by other workers. Hopkins [1920] has shown that when air is bubbled through butter for four hours at 120° the fat-soluble factor becomes inactivated. Drummond and Coward [1920] have also demonstrated that the destruction of this factor takes place in butter-fat in presence of air rapidly at high temperatures and more slowly at lower temperatures. Hess [1920] and Hess and Unger [1921] found that milk incubated with hydrogen peroxide lost its anti-scorbutic properties, whilst orange juice, milk, and tomato juice treated with oxygen lost some of their anti-scorbutic potency. Dutcher, Harshaw, and Hall [1921] have since confirmed Hess and Unger's observations concerning the destruction of the anti-scorbutic factor by hydrogen peroxide and the writer's observation on the stability of this principle at high temperatures in the absence of air.

The object of this communication is to describe a series of experiments on the action of ozone and air on the three accessory factors, observations having been made at various temperatures. These experiments owe their origin to the fact that the inactivation of the fat-soluble factor in butter by exposure to a mercury-quartz lamp [Zilva, 1919] was found by the author to be due to the action of the ozone generated by the lamp and not to the action of the ultra-violet rays.

The influence of ozone and air on the fat-soluble factor.

In the preliminary communication [Zilva, 1920] it was pointed out that when cod liver oil was exposed to the mercury-quartz lamp for 16 hours in the absence of air the activity of the oil was not destroyed by the action of the light. On the other hand six hours of exposure of the same oil to ozone in the dark entirely inactivated it.

In testing the above oils for the fat-soluble factor 2 g. of the oil were added to the daily basal diet of 20 g. when the rats had ceased to grow owing to the deficiency in the diet. The following are the results obtained:

With the addition of oil exposed to the mercury-quartz lamp in the absence of air.

Rat I ♀ gained 30 g. in 17 days.
Rat II ♀ gained 24 g. in 17 days.
Rat III ♀ gained 39 g. in 17 days.

With the addition of oil exposed to ozone for six hours in the dark.

Rat I ♂. No gain in weight in four weeks. On addition at the end of that time of a similar dose of the unexposed oil the animal resumed growth and gained 37 g. in three weeks.

Rat II ♂. No gain in weight in four weeks. On addition at the end of that time of a similar dose of unexposed oil the animal resumed growth and gained 23 g. in 16 days.

Rat III ♂. Declined in weight and died 26 days after the commencement of the administration of the oil. Developed keratomalacia during the period of treatment with the oil.

Rat IV ♀. No gain in weight in four weeks. On addition at the end of that time of a similar dose of unexposed oil the animal resumed growth and gained 29 g. in three weeks.

From the above experiments it is quite evident that ultra-violet light as such has no deleterious action on the fat-soluble factor. On the other hand ozone does inactivate it. Moreover the action of ozone must be very drastic, when one bears in mind that cod-liver oil is very potent. The oil employed in the above experiments has been found capable of inducing growth in rats in daily doses of 1·7 mgm. The experimental rats on the exposed oil consumed 60–70 % of their diet, in other words 1·2–1·4 g. of the oil or about 750 times as much as the minimum dose necessary to induce growth, without showing any tendency to grow. Possibly a much shorter exposure would have also produced inactivation of this factor.

In the earlier part of this work whale oil was used instead of cod-liver oil. The former is much less active than the latter and was given up for that reason. At the time the experiments were carried out it was not definitely settled that the fat-soluble factor was associated with the unsaponifiable fraction of the oil and it was therefore considered advisable to study the change in the iodine value of the oil during exposure to the mercury-quartz lamp. The differences observed were of a low magnitude. The following are the figures of a representative experiment:

Iodine value of whale oil before exposure	119
Iodine value of whale oil exposed to the lamp for eight hours in the absence of air	117·6
Iodine value of whale oil exposed for eight hours in the presence of the generated ozone	114·5

The change is not significant and the results are analogous to those obtained by Hopkins [1920] with butter heated in a stream of air at 120°. No

change was recorded in the iodine value of the butter after the treatment. It is very doubtful whether a chemical change such as the iodine value of the oil can have any bearing on the accessory factor.

Some experiments in connection with the inactivation of the fat-soluble were carried out in connection with another inquiry. It was necessary to inactivate a large batch of very potent cod-liver oil and Hopkins' method was adopted for that purpose. This method has an advantage over the employment of ozone because it does not change the consistency of the oil to such a great extent. Air was aspirated through the oil heated at 120° for 18 hours. By this means a very active oil was inactivated to the extent that a daily dose of about 0·75 g. did not induce any growth in rats.

The influence of ozone on the water-soluble factor.

Autolysed yeast juice was employed as a source for the water-soluble factor in this experiment. The juice was exposed to the ozone with constant shaking in the same apparatus and for the same time as the cod-liver oil. After the exposure the juice was very much altered in taste, smell and colour. It was incorporated in the basal diet free from the water-soluble factor after the rats had been on the restricted diet between three and four weeks. Three animals received 1, 2 and 3 cc. of autolysed yeast juice in their daily basal diet of 20 g. All the three animals responded by resuming growth, gaining 19, 24 and 15 g. respectively in 17 days. As 1 cc. of autolysed yeast juice is not much above the minimum dose capable of inducing growth there could not have been much destruction of the water-soluble factor in the juice by this very drastic method and we may conclude that this factor is decidedly more resistant to oxidation than the other two accessory factors. This resistance explains why on exposing autolysed yeast juice in a Petri dish to a mercury-quartz lamp no inactivation of the water-soluble factor takes place [Zilva, 1919]. Whether drastic oxidation inactivates the water-soluble factor at higher temperatures still remains to be ascertained.

The influence of ozone and air on the anti-scorbutic factor.

The influence of ozone in the dark on the anti-scorbutic factor was next investigated. Decitrated lemon juice, namely lemon juice from which the organic acids are removed by neutralisation with calcium carbonate and which retains almost the entire anti-scorbutic potency of the original juice, was exposed to ozone in precisely the same way as the active oils and the autolysed yeast juice. Fresh batches were prepared every two or three days so that no deterioration could take place on storage. The treated juices, as well as the control untreated juices of the same batch, were administered to guinea-pigs which had been kept on a scorbutic diet of oats and bran and autoclaved milk (40 cc.) for 14 days. Daily doses of 3, 5 and 7 cc. were administered with the following results:

Guinea-pig I. Dose 3 cc. Commenced losing weight after three weeks. Declined gradually and died 44 days after the commencement of the experiment. At the post-mortem examination evidence of scurvy was found.

Guinea-pig II. Dose 5 cc. Declined and died 35 days after the commencement of the experiment. At the post-mortem examination evidence of scurvy was found.

Guinea-pig III. Dose 7 cc. Maintained its weight for 53 days. Chloroformed at the end of this period. At the post-mortem examination some evidence of scurvy was found.

A control dose of 3 cc. of the original decitrated juice was found to be adequate to prevent scurvy for 56 days; the animal was still growing when the experiment was discontinued.

One may conclude from the above experiments that the ozone inactivated the juice to a great extent. The writer has previously found [Zilva, 1919] that when decitrated lemon juice was exposed to the mercury-quartz lamp its anti-scorbutic potency was not destroyed or even impaired in spite of the generated ozone. This can be explained by the fact that the juice was exposed in a quartz tube and, unlike the butter in that experiment, was shielded almost entirely from the generated ozone. The anti-scorbutic, like the fat-soluble factor, is, therefore, inactivated by ozone but is not affected by ultra-violet rays.

In view of the results obtained in the above experiments it became of interest to ascertain whether less drastic oxidative treatment such as exposure to air would also inactivate the anti-scorbutic factor. Air was therefore aspirated through decitrated lemon juice for 12 hours. Batches of juice prepared every two or three days were employed. The following is the protocol of the experiment:

Guinea-pig I. Dose 3 cc. Just maintained its weight for 51 days gradually declining in health and showing symptoms of scurvy during this period. The animal was chloroformed owing to its distressed condition. At the post-mortem examination evidence of advanced scurvy was found.

Guinea-pig II. Dose 5 cc. Commenced declining in weight after 17 days. Chloroformed 48 days after the commencement of the experiment. At the post-mortem examination evidence of scurvy was found.

Guinea-pig III. Dose 7 cc. Gained 57 g. in 51 days. Chloroformed 51 days after the experiment commenced. Evidence of mild scurvy was found at the post-mortem examination in this case.

A control dose of 3 cc. of the original juice was found quite adequate to prevent scurvy for 51 days. The control animals were still growing when the experiment was discontinued.

This experiment affords evidence that the exposure of decitrated lemon juice to air for 12 hours diminishes its anti-scorbutic potency very appreciably.

The above observations suggested the possibility that the inactivation of the anti-scorbutic factor by heat may be due in part if not entirely to the

presence of air during the heating. Such a possibility was conjectured by Delf [1920] who found that orange juice and swede juice retained their anti-scorbutic potency to a marked extent after being heated for a considerable time at temperatures higher than 100°. In order to test this theory the following experiments were devised. Two lots of decitrated lemon juice were heated at 100° under a reflux condenser. One was heated for one hour during which time air was bubbled through. The second was heated for two hours, air being all the time rigorously kept out by bubbling carbon dioxide gas through the solution. In this case also fresh batches of decitrated lemon juice were employed every two or three days. The following results were recorded:

Decitrated lemon juice heated in air for one hour.

Guinea-pig I. Dose 1·5 cc. Commenced declining in weight after a month. Chloroformed 43 days after the commencement of the experiment. Evidence of scurvy was found at the post-mortem examination.

Guinea-pig II. Dose 4 cc. Commenced to decline after three weeks and died 41 days after the commencement of the experiment. Evidence of acute scurvy was found at the post-mortem examination.

Guinea-pig III. Dose 6 cc. Maintained weight for 36 days and then suddenly declined. Died 43 days after the commencement of the experiment. Evidence of scurvy was found at the post-mortem examination.

The same juice heated in an atmosphere of carbon dioxide for two hours.

Guinea-pig I. Dose 1·5 cc. Gained 122 g. in 74 days. Chloroformed. No evidence of scurvy was found at the post-mortem examination.

Guinea-pig II. Dose 4 cc. Gained 48 g. in 74 days. Chloroformed. No evidence of scurvy was found at the post-mortem examination.

Guinea-pig III. Dose 6 cc. Gained 138 g. in 74 days. Chloroformed. No evidence of scurvy was found at the post-mortem examination.

This experiment affords proof that the anti-scorbutic factor is quite stable when heated in the absence of air.

Decitrated lemon juice contains a considerable quantity of reducing sugar. On hydrolysis, however, the reducing capacity of the juice increases slightly, a reducing substance, most probably a sugar, being liberated. An experiment was devised to ascertain whether the anti-scorbutic potency is destroyed when the juice is hydrolysed under anaerobic conditions. Decitrated lemon juice was therefore hydrolysed on a water-bath under a reflux condenser for five hours with 2 *N* hydrochloric acid. The air was displaced during hydrolysis by carbon dioxide. After hydrolysis the solution was neutralised with sodium carbonate and tested out for its anti-scorbutic potency on guinea-pigs. A fresh preparation of lemon juice was hydrolysed every day for feeding purposes. The following results were obtained:

Guinea-pig I. Dose 1·5 cc. Declined after three weeks and died 32 days

after the commencement of the experiment. Evidence of scurvy at the post-mortem examination.

Guinea-pig II. Dose 1·5 cc. Declined after 23 days. Died 30 days after the commencement of the experiment. Evidence of scurvy at the post-mortem examination.

Guinea-pig III. Dose 1·5 cc. Declined after three weeks and died 26 days after the commencement of the experiment. Evidence of scurvy at the post-mortem examination.

Guinea-pig IV. Dose 3 cc. Gained 30 g. in 37 days. Chloroformed. At the post-mortem examination the animal was found in excellent condition, but showed evidence of extremely mild scurvy.

Guinea-pig V. Dose 3 cc. Gained 28 g. in 37 days. Chloroformed. At the post-mortem examination the animal was found in excellent condition showing no evidence of scurvy.

Guinea-pig VI. Dose 3 cc. Gained 16 g. in 37 days. Chloroformed. At the post-mortem examination the animal was found in excellent condition showing no evidence of scurvy.

Guinea-pig VII. Dose 5 cc. Gained 18 g. in 37 days. Chloroformed. At the post-mortem examination the animal was found in excellent condition showing no evidence of scurvy.

Guinea-pig VIII. Dose 5 cc. Gained 71 g. in 37 days. Chloroformed. At the post-mortem examination the animal was found in excellent condition showing no evidence of scurvy.

Guinea-pig IX. Dose 5 cc. Gained 30 g. in 37 days. Chloroformed. At the post-mortem examination the animal was found in excellent condition showing no evidence of scurvy.

No control was tested out with this experiment owing to some mishap, but numerous experiments performed by the writer with decitrated lemon juice show that the minimum dose capable of preventing scurvy in guinea-pigs is about 1·5 cc. The conclusion one can draw from this experiment is that the hydrolysis diminishes the activity of the juice, but in spite of the very drastic treatment very significant activity is retained. This further confirms the view that the anti-scorbutic factor is stable towards heat providing anaerobic conditions are observed.

Summary.

1. The fat-soluble factor was destroyed in cod-liver oil by exposing it to ozone in the dark at ordinary temperature and by bubbling air through it at 120°.

2. The exposure of autolysed yeast juice to ozone in the dark did not destroy the activity of its water-soluble factor to any appreciable extent.

3. The exposure of decitrated lemon juice to ozone destroyed its anti-scorbutic activity. This was also destroyed by passing air through the solution at ordinary temperature.

4. Ultra-violet rays had no deleterious action on the accessory food factors in the absence of air.

5. Two hours' boiling did not destroy the anti-scorbutic potency of decitrated lemon juice in an atmosphere of carbon dioxide, whereas one hour's boiling in the presence of air destroyed its potency almost entirely.

6. Hydrolysis of decitrated lemon juice with hydrochloric acid for five hours impaired the anti-scorbutic activity of the juice. The juice however retained its activity to a very considerable extent.

In conclusion I wish to express my best thanks to Professor Sensho Hata and Miss E. M. Low for help in some of the experiments in this inquiry. My thanks are also due to Dr J. S. Edkins for having kindly permitted me to use his ozone generator.

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X. AN IMPROVISED ELECTRIC THERMOSTAT CONSTANT TO 0·02°.

By SAMUEL CLEMENT BRADFORD.

From the Science Museum.

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In these days of elaborate physical apparatus it may be of interest to describe a simple thermostat which can be made easily from material at hand and is sufficiently constant for most purposes. The apparatus, shown in the photograph, was required for an investigation expected to extend over twelve months or more and needing only moderate constancy.

The principle of the stirrer, on which the success of the thermostat depends, was suggested by Mr S. Bradshaw, to whom the author is indebted, also, for much kind assistance in making the apparatus (Plate I). The stirrer comprises a solenoid, A, with an iron plunger, B, mounted on a glass tube carrying an ordinary cork bung, shown in dotted outline, at its lower extremity. The upper part of the glass shaft bears two rubber corks, C and D, that strike the left arm of the brass lever, E, whose right arm carries a cylindrical plumbago contact. As the stirrer reaches the lower end of its stroke, the cork, C, pulls over the lever, causing the plumbago contact to engage with a similar contact, F, above. The current from the 110 volt supply now flows through the solenoid, raising the plunger until the cork, D, throws the lever back again, breaking the circuit and allowing the plunger to fall. To render the lever stable in either position, it is made top-heavy by means of the weight, G, carried on a stout wire soldered to the lever near its pivot.

For temperatures up to 25°, a 16 c.p. carbon lamp is sufficient as a heater. For higher temperatures a more powerful lamp may be employed, or an auxiliary one continually alight. The brass parts of the lamp are protected from the water by a short piece of 1 inch rubber tubing.

As pointed out by Lewis [1922], the heater should be placed near the thermo-regulator, M, which is similar to an ordinary toluene thermo-regulator for gas. This was made from a stout glass test-tube of about 50 cc. capacity when sealed to about 3 inches of 4 mm. glass tubing. To this was joined about 6 inches of 1 mm. bore capillary tube, having a short wider tube at its open end. About 2 inches from the upper end of the capillary, a short piece of 4 mm. tube, N, was sealed at right angles. The wider tube, joining the capillary and bulb, was then bent through 180° near its junction with the bulb. The bulb contains toluene and some mercury which also fills the

capillary tube. The height of the mercury in the latter is adjusted by sliding, within the side tube, a short piece of capillary tube bearing a platinum contact at its sealed end. The joint between the side tube and its piston is closed by a slieve of rubber tubing. The other platinum contact is carried at the end of a piece of glass tube, O, drawn out so as to pass vertically within the wider end of the capillary tube and rest on the constriction, with the contact projecting into the capillary tube.

These two contacts are connected, by mercury seals, in series with a 4 volt accumulator and the magnetic cut-out for the heater. For the cut-out, a discarded high-resistance telegraph relay, P, was fitted with iron wire dippers and two mercury cups in circuit with the heater. However, almost any high-resistance electro-magnet and armature could be adapted. By using an accumulator to actuate the cut-out, there can be no fouling of the mercury in the thermo-regulator, and it is unnecessary to provide for an up-and-down motion of the upper platinum contact. The capacity of the accumulator is immaterial, as it can be charged while in use through a single lamp, the potential difference across its terminals remaining at 4 volts. Actually a 40 ampere-hour accumulator will last a month without recharging. The capacity of the bath is about 5 litres. A constant level is maintained by the Marriott's bottle shown on the left.

Had the temperature remained constant within 0·1°, expectations would have been fulfilled. Consequently, it was astonishing to find that the variations were limited to 0·02°. The observations recorded below extended over a period of nearly 24 hours. The maximum variation occurs within a short period corresponding to, but lagging behind, the heating cycle. By bringing the heater right up to the thermo-regulator, probably even this small variation would be diminished. The disadvantage of doing so, when working for very long periods, lies in the chance of the mercury in the cut-out being fouled on account of the greater number of breaks. During the period under observation only one reading was made differing by more than 0·01° from the mean, and this was only 0·05° greater.

The solenoid, $4\frac{1}{2}$ inches long, with a pasteboard tube, allowing $\frac{1}{16}$ inch clear space round the iron core, is wound with about eight layers of No. 27 single cotton covered wire insulated with shellac varnish. The core is made from gas pipe, $\frac{3}{4}$ inch diameter, the same length as the solenoid, turned down to about $\frac{1}{32}$ inch thickness. Or it could be made from sheet iron. The glass shaft passes through two rubber corks in the ends of the core and is supported by guides, Q and R, constructed of sheet brass. It is convenient to make the glass shaft in two pieces joined by a cork, shown at the level of the heating lamp.

The contact lever, E, is the only part of the stirrer needing careful construction. It is fashioned from 18 gauge sheet brass. The pivot bearing is a piece of brass tube, $\frac{1}{8}$ inch long, sweated through the lever at its centre of mass, and ground with a little emery and oil to fit smoothly over the shank of a

round-headed brass screw forming the pivot. The mass of the weight, G, is about 25 g. One end of the bearing abuts against a brass plate screwed to the base-board, the other against the flange of the screw, which is adjusted so that the lever just swings easily, without looseness, between the two stops, K and L, jacketed with rubber tube. The thin steel springs, H and J, are carefully bent so that their pressures increase or diminish very gradually and evenly throughout the stroke. They engage the under side of the lever and the lower one should project upwards to the level of the end of the upper one. The pressures of the springs are adjusted by means of the screws seen near their centres, and must be only sufficient just to absorb the energy of the lever as it is brought to rest against the stops. To prevent sparking at the pivot, with consequent roughening of the bearing, the current is conveyed to the lever through a short piece of flexible wire soldered to it, as shown in the photograph.

(*a*) Thermometer remote from thermo-regulator

θ	T h. m. s.
23·52°	3 5 0 p.m.
·51	7 0
·50	7 30
·51	8 0
·52	9 0
23·50	3 17 0
23·50	3 45 0
23·50	4 0 0
23·51	4 15 0
23·52	10 30 0 a.m.
23·52	10 50 0
·52	53 0
·53	54 0
23·515	11 5 0
23·52	11 39 0
·51	39 30
·505	40 0
·51	40 10
·52	40 20
·525	41 0
·52	41 20
23·52	12 26 0 p.m.
·51	26 30
·50	27 0
·51	27 20
·52	27 30

(*b*) Thermometer near thermo-regulator

θ	T h. m. s.
23·515°	12 30 0 p.m.
·505	30 30
·505	31 0
·515	31 30
·52	32 0
·515	32 10
·51	32 30
·51	33 0
23·51	1 10 0
·515	11 0
·51	11 20
·51	12 0
·505	12 20
·51	13 0
23·515	2 12 30
·51	12 50
·50	13 10
·50	13 30
·505	14 0
·51	14 10
·515	14 30
·51	14 50
·505	15 0

The induction in the solenoid causes a powerful spark at each break and a great deal of trouble was experienced with the contacts. When first set up, the apparatus worked for several hours, but the metal contacts used soon became corroded. Mercury contacts in oil lasted only about 48 hours. Finally,

cylindrical plumbago contacts, 8 mm. in diameter, as used for magneto brushes were adopted. The lower one fits firmly in a clip soldered to the lever. The upper contact slides tightly in a thin split metal cylinder, which fits loosely in a short vertical piece of glass tube, clamped to the base-board, and has a projection at the top to prevent the contact holder dropping through the glass tube. The flexible wire bringing the current is soldered to this projection. The position of the upper plumbago rod in its holder is adjusted so that, when the lever is in the contact position, the lower rod has raised the upper one about $\frac{3}{16}$ inch above its resting position. Thus, at each make and break, the lower contact pushes the upper one up and down, and upon this motion the efficiency of the apparatus appears to depend. Probably carbon contacts would serve nearly as well as plumbago ones.

Even with this arrangement, however, the apparatus could hardly have been regarded as satisfactory, owing to excessive sparking. Ultimately the device was adopted of allowing a small current, just insufficient to raise the plunger, to flow through the coil constantly. Only the extra current, necessary to actuate the stirrer, is passed through the contacts. With this arrangement, the coil is always on a closed circuit, and the smaller induced current, due to breaking the extra current, discharges through the closed circuit, almost entirely avoiding the spark. The constant current flows through an 8 C.P. carbon lamp, and the size of the bung is then adjusted so that its buoyancy is sufficient just not to float the plunger. The extra current passes through an 8 C.P. lamp or preferably, a 16 C.P. lamp with a resistance (which may be made of iron wire), about 150 ohms, adjusted so that the plunger moves regularly with the required power. The position of the corks, C and D, is adapted to the length of stroke, about $2\frac{1}{2}$ inches.

The only attentions the apparatus requires are to give a drop of oil to the pivot, the brass springs and the sliding contact holder, and to sand-paper the surfaces of the plumbago contacts about every 48 hours. Occasionally the pivot screw, or those of the springs, may need a fraction of a turn. The guides of the shaft are best lubricated with vaseline. At the time of writing the thermostat has been running smoothly for over a month.

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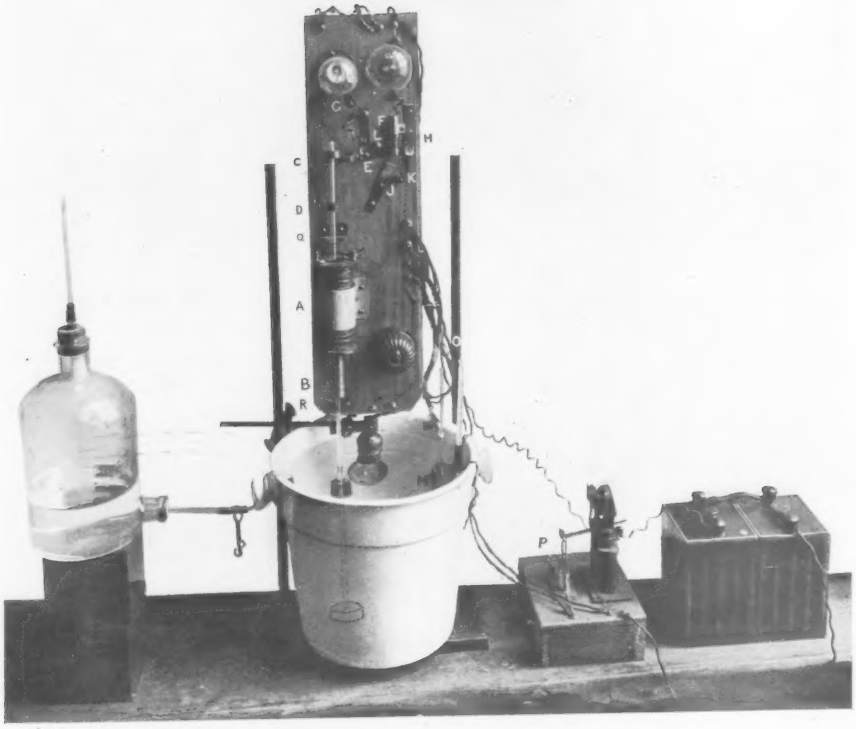
C
F
H
E
K
J
D
A
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P

XI. TETHELIN—THE ALLEGED GROWTH-CONTROLLING SUBSTANCE OF THE ANTERIOR LOBE OF THE PITUITARY GLAND.

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AND

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CERTAIN conditions of disordered development, such as acromegaly and gigantism, have long been associated with abnormal conditions of the pituitary body and in consequence the function of this gland in the growth of the animal has been the subject of much investigation. The suggestion that the pituitary body supplies a hormone which regulates the growth of the body, particularly of the skeleton, arose directly from the pathology of the diseases in question and has directed research along two definite lines. On the one hand studies of the effect of extirpation of the gland have been made, on the other, light has been sought in experiments based on the administration of the gland or its extracts to a normal animal.

The extirpation of one or other of the lobes of the pituitary body without injury to the remainder or to surrounding tissues is attended with extreme difficulty owing to the intimate association of the parts and, in consequence, results of such experiments have been conflicting. It is generally agreed that removal of the posterior lobe gives rise to no marked symptoms, whereas the balance of opinion points to the conclusion that with extirpation of the anterior lobe in young animals growth is checked, fatty deposits increase, metabolism is reduced and persistent infantilism results.

The results of experiments on the administration of the gland to normal animals are, again, far from unanimous. Cerletti [1907] injected pituitary emulsion into young animals and reported retardation of bone growth and Cushing [1909] by repeated injection of anterior lobe extracts obtained a fall in weight of young animals. The feeding of pituitary by Sandri [1909] to young guinea-pigs arrested growth whilst Schafer [1912] found that oral administration of anterior lobe was without appreciable effect on the growth and metabolism of young rats. Aldrich [1921] from experiments on puppies came to the same conclusion.

The general opinion at this period was, therefore, that oral administration of anterior lobe did not appreciably accentuate any regulating influence which the gland might normally have upon growth. The value of much of this earlier work is however depreciated by the fact that various forms of administration and different preparations of the gland were adopted by the different workers.

The whole question was reopened by a series of papers by Brailsford Robertson in 1916. He claimed to have demonstrated that the feeding of the fresh anterior lobe of the ox to white mice leads to a "marked retardation of growth in the beginning of the third growth cycle (6–10 weeks) followed by a pronounced acceleration from the 20th to 60th weeks" [1916, 1]. Further, he isolated from the anterior lobe of the ox a substance to which he gave the name *tethelin*, and which he regards as the active growth-controlling principle [1916, 2].

We were, at first, impressed by the care with which Robertson's feeding experiments had been carried out and it did appear that, by the employment of large groups of animals and by a statistical control of his data, he had guarded against all sources of error common to such experiments. A closer inspection of his work, however, revealed many inconsistencies which in our opinion seriously detract from the validity of his conclusions. In particular, it appeared that nothing but a substance of very impure character could possibly be obtained by the method he described for the isolation of tethelin [1916, 3].

The communications referred to were followed by others in which the chemical and physiological properties of tethelin were described and its effects in increasing the rate of tumour growth [1916, 4], in accelerating the healing of wounds and recovery after inanition [1916, 5] were reported. Meanwhile other workers were obtaining varying results. Goetsch [1916] and Marinus [1919] independently observed accelerated growth in rats and Wulzen [1916] accelerated division of planarian worms whilst Pearl [1916] obtained retarded growth of chickens fed upon anterior lobe.

The conflicting nature of all these results is not dispelled by Robertson's later papers in which, from a consideration of his accumulated data, he modifies his former views and comes to the conclusion that the influence of tethelin upon growth consists, as far as the whole animal is concerned, in retardation. His latest explanation of his former conclusions and the development of these views into a theory of growth catalysers is ingenious but quite unconvincing [1919, 1921]. We were led by the conflicting nature of all the evidence to undertake the re-investigation the results of which are reported in this paper. For the opportunity to carry out the work we are indebted to Mr T. O. Kent, who most kindly supplied us throughout with quantities of fresh anterior lobe of the pituitary of the ox.

Isolation of tethelin.

The method for the isolation of tethelin described by Robertson [1916, 3] is briefly as follows. The mixed anterior lobes dried by intimate mixture with an anhydrous inorganic salt are extracted for 48 hours with boiling absolute alcohol. The extract is concentrated *in vacuo* until separation of a precipitate begins. After cooling, one and one half volumes of dry ether are added and a voluminous white precipitate separates, which, after three hours, is filtered, washed with alcohol-ether mixture and dried in an incubator at 30–35°. Robertson appears to claim chiefly from constancy of nitrogen and phosphorus content that the substance is a chemical individual. To anyone familiar with such methods for the extraction of tissues it must be obvious that only a crude product, consisting largely of lipoid substances would be yielded by his technique and, further, that no such product would preserve its characteristics unless carefully protected from oxidation throughout the preparation and drying. We have made several attempts to prepare this substance and to obtain a white precipitate such as Robertson describes by following the detail of his method. Our yield varied from 0·4 to 0·8 % of the moist gland, but the products obtained did not show constancy of nitrogen and phosphorus content as reported by Robertson. The analyses of two samples which agree in their content of nitrogen are given in Table I. In all cases our preparation was carried through in an atmosphere of carbon dioxide.

Table I. *Analyses.*

	Our preparations			
	I	II	Robertson	Protagon
Nitrogen	2·88	2·80	2·58	2·39
Phosphorus	1·76	0·81	1·41	1·07

Our preparations resembled in many ways the mixture of lipoid substances which one can prepare from animal tissues by following a method essentially that of Robertson, and the analytical figures as well as the appearance and properties of the products show a resemblance to admittedly impure mixtures of this type such as protagon. Robertson described a number of properties of tethelin which are typical of such lipoid mixtures but we were unable to confirm the colour reactions upon which he is rash enough to suggest the presence in the molecule of inositol and of an iminazolyl radicle.

On the assumption that tethelin represents an impure mixture of substances of the lipoid class, perhaps contaminated by other cell constituents—no purification of his product appears to have been attempted by the author—we submitted our preparations to a simple fractionation. By extraction with hot alcohol they could be divided into a fraction readily soluble in that solvent and an insoluble fraction.

The soluble fraction deposited, on standing, a white pseudo-crystalline product of which 1·68 g. were obtained (T_1). The mother liquor of T_1 yielded

on evaporation a sticky brown residue, T_3, whilst the fraction insoluble in alcohol was designated T_4. On analysis these products gave the figures:

	T_1	T_3	T_4
Nitrogen	1·63	3·43	3·23
Phosphorus	0·88	1·54	3·94

We endeavoured to carry the fractionation further but were led to abandon it owing to the small amount of material at our disposal and to our conviction that the matter was not worthy of further consideration.

We did, however, examine the fraction T_1 which appeared to have the properties of an impure mixture of galactosides.

A determination of the melting point as described by Rosenheim [1914, 1] showed the following:

	Phrenosin (Rosenheim)	T_1
Softens	130–140°	—
Shrinks	170–190°	190°
Darkens	—	209°
Melts	205–215°	214°

In order to determine if the substance were mainly galactosides we hydrolysed 0·5 g. of T_1 by the method described by Rosenheim [1913]. 0·34 g. of impure esters was obtained and this upon hydrolysis yielded 0·23 g. of fatty acids, having a melting point of 58°. Recrystallisation from alcohol gave a crop of white needles melting at 70° which after a second recrystallisation melted at 76°.

A few oily drops were separated from the products of hydrolysis of T_1 and may have been the sulphates of the bases, but there was insufficient for examination.

The hydrolysate, freed from esters, was examined in the polarimeter. The rotation, if any, was slight and laevo in direction. A minute amount of a methylphenylosazone which melted at 182° was obtained (methylphenylgalactosazone M.P. 190°). A further amount of T_1 was extracted with pyridine following the method of Rosenheim [1914, 2] and yielded fractions of varying nitrogen and phosphorus content. There was insufficient of these for further examination.

There appeared, from all the data, to be little doubt that the product obtained by following the method described by Robertson for the isolation of tethelin is a very impure mixture of substances of the lipoid class and that this product is capable of separation by simple means into fractions of variable composition.

Feeding experiments.

Robertson appears to be well aware of the high variability of the growth curves of white mice but it is his claim that, by the employment of large groups of animals, a composite growth curve can be constructed which will to a high degree of probability reproduce the characteristics of growth of the individual. On this statistical basis he feels entitled to draw definite and far reaching conclusions from small differences in such composite curves for

normal and tethelin-fed groups. An examination of his methods and data led us to query the justice of this argument.

For example, it is significant that, in his experiments on the effect of feeding egg-lecithin [1916, 6], two batches of normal mice chosen at random at the age of four weeks showed a difference of 1·5 g. in mean weight, *i.e.* 13 % of body weight—a difference of the same order as the defection of weight of tethelin-fed mice at the maximum divergence of this group from the normal. Yet this, in the former case, is explained away as being due merely to the high variability at that age, whilst in the latter case it is reported as being a marked defection due to the effect of tethelin. Many other examples of such biassed interpretation occur throughout this series of papers but one further example will suffice. In support of his contention that mice fed upon pituitary are, size for size, heavier than those normally fed. Robertson reproduces a photograph of two mice of the same age and linear dimensions, one a normal mouse weighing 30 g. the other a pituitary-fed mouse weighing 37 g. [1916, 1]. When it is pointed out that the weight of the normal was about the mean for his batch whereas the other weighed 27 % more than the mean for his batch—*i.e.* was an extravagantly abnormal member—the fallacy of the argument is apparent.

On the basis of such criticisms we submitted the author's papers to Professor Karl Pearson under whose direction, Mr H. Soper, of the Department of Applied Statistics, University College, London, undertook the examination of Robertson's statistical arguments, and who has kindly permitted us to publish extracts from his report. He says "if some mice are apt to be 3 days in advance of or behind the others in stages of growth as seems indicated, then the curve of mean growth will not reproduce the characteristics of individual growths. The addition of curves at different epochs or phases will tend to distort or obliterate the detail." It is significant that just such a possible variation in stage of growth was introduced into the experiments by the empirical grouping together of animals for weighing. Thus animals weighed between the 25th and 31st days inclusive were regarded as having been weighed upon the 28th day. A similar bracketing of ages was employed throughout. Mr Soper sums up in these words: "The conclusion to be drawn is that on the figures presented and statements made there is a significant defect in weight in all the treated groups from the normal group. The normal group was not changed and was not large in numbers and it has been assumed that it was a random sample of the whole community under observation. The figures given are not so complete as they might have been and statements are made and procedures taken which could only be supported by data not detailed."

It is significant that the normal group was not strictly a "random sample of the whole community under observation" as their growth curve antedates that of the pituitary mice by three weeks and that of the tethelin mice by three months. The stages of growth of the contrasted batches were not con-

temporary and there is no evidence that undetected variations in environment incident to the two groups at different ages were not responsible for the differences in growth. Robertson himself [1919] has acknowledged that the normal growth curve varies year by year.

Any experiment, claiming acceptance upon statistical considerations, which in the methods employed introduces a possibility for variability from that statistical basis must be open to criticism. Finally an inherent source of error vitiates all the conclusions. The animals were in possession of their own functioning pituitary glands, the activity of which could be neither measured nor controlled.

Experimental.

It was not possible with the material at our disposal, nor in view of our previous findings was it considered worth while, to carry out feeding experiments on the scale of those of Robertson, but all the other precautions employed by him in respect to weighing, elimination of sickly mice and general care were taken and the errors which it is suggested existed in his methods have as far as possible been avoided.

The mice were placed as soon as weaned in groups of six of the same sex in special wooden boxes and fed on a basal diet of mixed grains and dry bread with meat and vegetables once a week and water *ad libitum*. To the treated animals dried anterior lobe was administered in a dose of 50 mg. per mouse per day (equal to about 3 mg. of tethelin). This was given in a paste of dried milk powder and starch in equal parts and was greedily consumed before the day's ration of ordinary food was given. The same quantity of the paste, without pituitary, was given to the normal animals.

In the first experiment mice were taken at an age between 3–5 weeks and divided into batches of 25 normal and 25 pituitary-fed mice of each sex. The individual growth curves were plotted until maturity. In view of the uncertainty of age no composite growth curves were constructed but it was thought that a careful inspection of such groups of individual curves would reveal the general characteristics of the two rates of growth and the distortion of the normal curve found in Robertson's tethelin curve would be detectable. No general tendency towards a differentiation of the shapes of the two batches of curves was to be observed, a close inspection merely emphasising the high variability of growth.

In a second experiment mice were observed from birth so that composite curves might be drawn. The mothers of the treated mice were fed with pituitary until the young were weaned, when the latter were removed and treated as in the first experiment. The experiment was abandoned at 13 weeks owing to pituitary supply having become exhausted but as Robertson reports marked effects from 7 weeks onwards a comparison was possible.

These growth curves show no characteristic differences. Admittedly the curves are built up from a statistically too limited number of mice but, in conjunction with the other work reported their negative result is significant.

Age	Male mice (ten in each group) Mean weight. g. Normals	Male mice (ten in each group) Mean weight. g. Pituitary-fed	Female mice (ten in each group) Mean weight. g. Normals	Female mice (ten in each group) Mean weight. g. Pituitary-fed
Birth	1·60	1·44	1·53	1·50
1 week	3·92	4·20	4·00	4·10
2 weeks	5·55	5·90	5·80	5·75
3 „	7·44	8·00	8·02	8·30
4 „	9·70	10·0	10·1	10·1
5 „	12·0	12·1	12·4	12·0
6 „	14·3	14·0	14·3	13·4
7 „	15·1	15·6	15·4	14·8
8 „	16·0	16·9	16·4	16·0
9 „	17·2	18·0	17·6	17·6
10 „	18·7	19·5	19·0	18·4
11 „	19·8	20·4	19·6	19·0
12 „	21·1	21·4	20·3	19·8

Conclusions.

1. The product obtained by the method described by Robertson for the isolation from the anterior lobe of the pituitary gland of the substance he has called *tethelin* is a very impure mixture of substances of the lipoid class.

2. Criticisms are advanced of the deductions made by Robertson as to the effect of tethelin and of anterior lobe of the pituitary gland upon growth and experiments are reported which fail to point to any influence upon the growth of mice of oral administration of anterior lobe of the pituitary gland.

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XII. THE ESTIMATION OF PECTIN AS CALCIUM PECTATE AND THE APPLICATION OF THIS METHOD TO THE DETERMINATION OF THE SOLUBLE PECTIN IN APPLES.

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In order to follow the changes which take place in the pectic constituents of stored apples, a number of preliminary investigations have been found to be necessary. In the first place a method was required by which pectin could be estimated accurately in dilute solution. This having been established, it has become possible to proceed to an investigation of the means by which the various pectic constituents of the fruit can be separately extracted. The present communication, in so far as the extractions are concerned, is confined to an examination of methods for extracting the soluble pectin of fruits. The difficulties which are encountered in the course of this extraction are almost entirely mechanical—difficulties inseparable from the process of washing out this soluble colloidal material from the insoluble colloids of the cell wall with which it is intimately associated. The clearing up of these mechanical difficulties is a necessary preliminary to any further investigation of methods of extraction, for unless suitable methods of washing out are adopted much soluble pectin may remain behind to form a mixture with the pectin dissolved out by subsequent treatment.

It has been the general practice hitherto to estimate the pectin content of solutions by precipitating the pectin with alcohol. This method at its best is inconvenient and lacking in accuracy. The pectin obtained is necessarily of variable composition, since pectin probably exists in a number of forms intermediate between neutral pectin and pectic acid. Moreover, as dilution increases precipitation becomes increasingly difficult, until at very low concentrations no precipitate is obtained, even after prolonged standing with a large excess of alcohol. By the method now adopted the difficulty of precipitation is avoided, for calcium pectate can be made to flocculate from solutions of very low concentration, and a product of definite chemical composition is obtained. A comparison of the results of precipitating pectin with alcohol and as calcium pectate is given in the sequel.

The precipitation of calcium pectate.

Since calcium pectate is a colloid, its state of aggregation varies greatly with the conditions under which it is precipitated, and in order to obtain a product which can be easily manipulated it is requisite to adjust these conditions carefully. Great difficulties were experienced in the early stages of this investigation by the occurrence of a sticky condition in which the precipitate could only be washed and filtered with extreme difficulty. This has been found to be the result of precipitating in alkaline solution, in which case calcium hydroxide is absorbed by the gel [Haynes, 1914]; if the period of hydrolysis with sodium hydroxide is too prolonged, or if the alkali is too concentrated, a similar result tends to be produced. On the other hand, too small an excess of alkali above the theoretical amount produces unsatisfactory coagulation, and as the concentration of pectin is increased the excess of alkali required increases also. In the opinion of the writers, both effects are probably a consequence of the tendency which alkalies exhibit to replace the loosely combined water molecules of the pectin sol.

To avoid these difficulties the precipitation of calcium pectate is carried out in the following stages:

(1) Hydrolysis with sodium hydroxide.
(2) Acidification with acetic acid.
(3) Addition of calcium chloride.

Since no independent method exists for checking the results of pectin estimations, it has been necessary to carry out series of experiments and to ascertain how the weight of the calcium pectate varies when variations are made from a standard procedure arbitrarily adopted. In most cases determinations of the ash content of the precipitate calculated as calcium were also carried out. Since the number of interdependent factors is considerable, this has involved a large amount of work. No useful purpose would be served by giving a complete account of the details of this preliminary investigation, but some remarks on the successive stages of the process are required, and the results of a few experiments will be quoted in support of the statements made. The results of ash determinations require more detailed discussion, and will be treated separately below. The pectin solutions used for the investigation were in almost every case obtained from apple. Many determinations were made directly on the liquid obtained by expressing and washing out apple pulp, previously killed by freezing; but confirmatory series were also carried out on pectin from a similar source which had been precipitated by alcohol, re-dissolved and allowed to stand under toluene until suspended impurities were deposited. The juice in each case was heated immediately after pressing, to destroy pectase. It was found convenient to work with a quantity of solution giving from 0·02–0·03 g. of calcium pectate, and to dilute to a concentration of approximately 0·01 % before precipitation. Larger quantities

give so great a bulk of precipitate that it is difficult either to wash thoroughly or to dry satisfactorily.

Hydrolysis. Fellenberg [1918] has stated that pectin hydrolyses very rapidly in the presence of a small excess of alkali. The following series show the general results obtained in the course of the present investigation.

	Weight of calcium pectate obtained			
	Series I (Concentration of NaOH) *N*/100		Series II (Concentration of NaOH) *N*/50	
Time				
10 mins.	Very little		0·029	0·026
30 ,,	0·031	0·0285	0·0315	0·032
60 ,,	0·028	0·0285	0·0305	0·0305
4 hrs.	0·028	0·028	—	—
24 ,,	0·028	0·028	0·0295	0·030
48 ,,	0·028	0·0275	—	—

It will be observed that the rate of hydrolysis increases very rapidly with the concentration of alkali. As it is advisable to keep this concentration low, it is necessary to allow the mixture to stand at least an hour. The best results have been obtained by leaving it overnight, in which case the precipitate flocculates readily and is easy to wash and filter. It is noticeable that rather high results were obtained in both series for the half-hour period. This is a very frequent result of incomplete coagulation which renders effective washing extremely difficult.

Acidification and addition of calcium salt. Calcium pectate can be boiled with water without change, but with dilute acids it tends to pass into colloidal solution. The conditions of this change have not been fully investigated, but it appears that prolonged boiling reverses the peptising effect of acid. This is shown by the results of the following series of experiments carried out on equal quantities of pectin precipitated as calcium pectate.

Treatment		Weight of calcium pectate	
Boiled with water		0·0275	0·028
Boiled with *N*/2 acetic acid	5 mins.	0·0205	0·0225
,, ,, ,,	30 ,,	0·0215	0·024
,, ,, ,,	60 ,,	0·026	0·0265
Boiled with *N*/10 acetic acid	5 ,,	0·0245	0·027
,, ,, ,,	30 ,,	0·0285	0·028
,, ,, ,,	60 ,,	0·026	0·023

The precipitates which had been boiled with acid were found to contain a low percentage of ash. The action of acid must therefore be primarily attributed to the decomposition of calcium pectate; this being so, it is not surprising to find that an excess of calcium salts tends to reverse the effect and that calcium pectate may be boiled with dilute solutions of weak acids in the presence of a sufficient excess of calcium chloride without change of weight. The amount of calcium salt required increases very rapidly with the amount of pectin present. This is shown by a comparison of the two following series of precipitations:

Precipitation carried out with: 100 cc. $N/10$ NaOH; 100 cc. $N/1$ acetic acid; x cc. $M/1$ $CaCl_2$:

Cc. $M/1$ $CaCl_2$ added	Series I 200 cc. apple pectin (1)	Series I 200 cc. apple pectin (2)	Series II 400 cc. apple pectin as used in Series I (1)	Series II 400 cc. apple pectin as used in Series I (2)
2	0·0300	0·0300	0·0565	0·0510
5	0·0300	0·0290	0·0560	0·0575
10	0·0295	0·0300	0·0600	0·0555
25	0·0305	0·0310	0·0605	0·0600
50	0·0300	0·0300	0·0605	0·0600
100	—	—	0·0605	0·0615

Effect of acid concentration.

Precipitation carried out with: 100 cc. $N/10$ NaOH; x cc. acetic acid; 50 cc. $M/1$ $CaCl_2$:

Cc. acetic $N/10$	Series I 200 cc. dilute apple pectin (1)	Series I 200 cc. dilute apple pectin (2)	Series II 400 cc. dilute apple pectin as in I (1)	Series II 400 cc. dilute apple pectin as in I (2)
2	0·1042	0·1270	—	—
10	0·0375	0·0365	—	—
20	0·0300	0·0305	—	—
50	0·0305	0·0290	—	—
100	0·0300	0·0295	—	—
$N/1$				
15	0·0310	0·0290	0·0645	0·0660
30	0·0300	0·0295	0·0615	0·0620
50	0·0300	0·0300	0·0610	0·0615
100	0·0300	0·0300	0·0605	0·0610

It will be seen that in the more concentrated series II the lowest limit of acid is only reached at 50 cc. of $N/1$ acetic acid, whereas in series I, 20 cc. $N/10$ acetic acid are seen to be sufficient. The higher values in both series are due to the carrying down of calcium carbonate and occluded impurities which are only removed by excess of acid.

It appears from these results that for ordinary concentrations of pectin the optimum concentration of calcium chloride is approximately $M/1$, and of acid $N/5$. Where the quantity of pectin is very small it has been found advisable to reduce the acid concentration to $N/10$, since the larger amount tends to produce stickiness. In this case the quantity of calcium chloride should be correspondingly reduced.

Method of estimating pectin as calcium pectate.

As a result of these investigations the following method of estimating pectin has been adopted:

A quantity of pectin is taken which will yield from 0·02 to 0·03 g. of calcium pectate; this is neutralised and then diluted to a volume such that after addition of all reagents the total volume measures about 500 cc. 100 cc. of $N/10$ NaOH are then added and the mixture is allowed to stand at least an hour, but preferably overnight. 50 cc. of $N/1$ acetic acid are then added, and after five minutes 50 cc. of $M/1$ calcium chloride. The mixture is then allowed to stand for an hour, after which it is boiled for a few minutes and filtered through a large fluted filter. If the precipitation has been properly carried out, filtration should take place very rapidly and subsequent washing

should be easy. The washing is continued with boiling water until the filtrate is free from chloride, after which the precipitate is washed back into the beaker, boiled, and filtered again. It is then again tested for chloride, and these processes are repeated until the filtrate from the boiled precipitate gives no indication of chloride with silver nitrate. It is then filtered into a small fluted filter, from which it can be transferred to a dish and finally to a Gooch crucible which has been previously dried at 100°. The precipitate is dried to constant weight at 100° which has been found to require about 12 hours.

If the quantity of pectin is increased, the quantities of soda and calcium chloride should be correspondingly increased. If very small quantities of pectin are dealt with, the acid and calcium chloride should be reduced.

Example of estimations using dilute apple pectin.

Precipitations carried out with: 50 cc. pectin solution; 50 cc. $N/10$ soda; 100 cc. $N/10$ acetic acid; 100 cc. $M/1$ $CaCl_2$:

Series I Purified pectin solution		Series II Unpurified pectin solution	
0·0236	0·0237	0·0280	0·0285
0·0237	0·0236	0·0287	0·0300
0·0240	0·0231	0·0265	0·0275
0·0240	0·0230	0·0280	0·0280
0·0229	0·0233	0·0280	0·0285
Mean = 0·0235		Mean = 0·0282	

The quantity of acid used for these estimations is less, and the quantity of $CaCl_2$ greater, than prescribed in the text. It has been found that the alteration of these amounts facilitates the washing of the precipitate.

If precipitated according to the method given above, calcium pectate is so insoluble that quantitative precipitation can be carried out in solutions of extreme dilution, the limiting factors being rather those governing the drying and weighing of very small quantities of colloidal material. It is thus possible to carry out estimations by the calcium pectate method over a large range of concentrations at which precipitation by alcohol is impossible. The lower limit of concentration at which pectin can be even partially precipitated by alcohol has been found to be 0·06 %.

Example of method applied to very dilute solutions.

Precipitations carried out with: 50 cc. dilute pectin solution (apple extract); 50 cc. $N/10$ NaOH; 50 cc. $M/1$ $CaCl_2$; 50 cc. $N/1$ acetic acid:

Weight of calcium pectate	0·0053	0·0055
	0·0050	0·0055
	0·0050	0·0053
	0·0055	0·0050
	Mean = 0·00525	

A few examples of estimation by precipitation with alcohol are given below for comparison. They are all carried out on similar quantities (10 cc.) of apple juice to which a small quantity of water extract has been added.

	Weight of precipitate (1)	(2)
25 cc. of alcohol	0·019	0·021
50 „ „ (1)	0·018	0·015
(2)	0·012	0·015
100 „ „	0·018	0·020

The weights of calcium pectate obtained from 10 cc. of this extract were: (1) 0·023; (2) 0·023. It is probable that all the alcohol precipitations are too low. The solubility of pectin in alcohol is shown clearly by the following series of results, carried out on the same material as above:

(1) The weight of precipitate obtained by adding 50 cc. of alcohol to 10 cc. of extract diluted with 25 cc. of water was found to be (1) 0·019; (2) 0·020.

(2) With 50 cc. of water no precipitate was obtained in two days in either sample. After this a small precipitate was obtained from one, but not from the other.

(3) With 100 cc. of water no precipitate was obtained after standing for a week or even after doubling the amount of alcohol.

These experiments have not been repeated on purified pectin owing to lack of material. Such experiments would probably give more regular results, since in ordinary juice sugar acids and other substances are present which may themselves tend to favour coagulation.

In the foregoing account it has been assumed that the method of pectin estimation there described is dependent upon the production of a definite chemical compound—calcium pectate. This assumption obviously needs justification, more especially since pectin is a colloidal substance which is likely to form adsorption compounds and is known to form solid solutions with alkalies and akaline earths [Haynes, 1914].

Pectic acid was among the first of the pectic compounds to be described; indeed the formation of pectic acid by hydrolysis still serves to define the class of substances known as pectins. The chemical nature of pectic acid is still obscure, but it is universally recognised as possessing definite acidic properties, and it has been found to possess the same chemical composition expressed by the empirical formula $C_{17}H_{24}O_{16}$ [Schryver and Haynes, 1916][1] when derived from very different sources (strawberry, turnip, and rhubarb stalks).

The pectic acid from apples alone showed a slight variation, and the present work indicates that this may very possibly have been due to the presence of impurities.

Fellenberg [1918] assigns the formula $C_{62}H_{96}O_{52}(CO_2CH_3)_8$ to pectic acid on somewhat arbitrary grounds, and his own analyses of barium salts agree more nearly with the empirical formula given above.

The number of decomposition products obtained by Ehrlich and others from pectin indicates, however, that the molecule of pectin is large, and it is therefore hardly likely that pectic acid can be represented by a simple multiple of $C_{17}H_{24}O_{16}$. It probably differs from a simple multiple by some small

[1] In this paper the name pectin was used as the equivalent of pectic acid. There is however an increasing tendency to reserve the term pectin for soluble products, especially those occurring naturally in fruits, and to denote by pectic acid the last term in a series of pectic compounds obtained by hydrolysis of these soluble substances. This usage is advocated by Fellenberg, and is followed in the present paper.

quantity but the true formula cannot be ascertained with any certainty until the constitution of pectic acid is established; in the meantime it seems advisable to adopt with reservation the formula given above, and it is to this formula, assumed to be that of a dibasic acid that the results of the analyses given below are referred.

THE COMPOSITION OF CALCIUM PECTATE OBTAINED FROM APPLES.

Two methods of analysis have been employed:

(1) Ignition to CaO.

(2) Conversion of this to sulphate.

The following results were obtained by the ignition of calcium pectate obtained from a purified solution of apple pectin which contained at most 0·01–0·03 % of ash when precipitated by alcohol. When filtered carefully before precipitation the amount of ash was negligible.

Weight of Ca pectate	Weight of CaO	Percentage Ca
0·0160	0·0017	7·59
0·0226	0·0025	7·90
0·0270	0·0029	7·66
0·0266	0·0028	7·51
0·0746	0·0080	7·65
0·0308	0·0033	7·64
		Mean = 7·658

In order to afford a comparison of the results of the two methods of ash determination, the following series are given. These were carried out on material similar to that used for the previous series. The calcium pectate was obtained by exactly similar methods of estimation in each case.

As CaO			As $CaSO_4$		
Weight of Ca pectate	CaO	Percentage Ca	Ca pectate	$CaSO_4$	Percentage Ca
0·0746	0·0080	7·65	0·0240	0·0062	7·60
0·0266	0·0028	7·52	0·0250	0·0065	7·65
0·0270	0·0029	7·60	0·0236	0·0062	7·70
0·0260	0·0017	7·59	0·0234	0·0062	7·65
0·0226	0·0025	7·70	0·0240	0·0064	7·67
		Mean = 7·612			Mean = 7·654

Mean of the two series = 7·63.

It is evident that these results agree very closely with the theoretical percentage, 7·66, and the same percentage has been obtained repeatedly from calcium pectate precipitated under widely different conditions, it may therefore be regarded as established that calcium pectate obtained by this method is a definite chemical compound, the empirical formula of which approximates closely to $C_{17}H_{22}O_{16}Ca$.

Since estimations of pectin have usually to be made in a water extract containing sugars, salts and other compounds, it has been necessary to ascertain how nearly the composition of the precipitate from this unpurified material corresponds with theory. It has been found that the ash is usually

rather high, but that if sufficient precautions are taken the error can be reduced to an amount which probably seldom exceeds 1 % of the total ash.

The following are representative examples, each set being estimated on different material:

	Ca pectate	$CaSO_4$	Percentage Ca
I	0·0243	0·0062	7·50
	0·0293	0·0076	7·63
II	0·0553	0·0144	7·66
	0·0577	0·0173	8·30
	0·0294	0·0077	7·70
III	0·0514	0·0138	7·90
	0·0151	0·0040	7·79
	0·0503	0·0129	7·54
	0·0425	0·0111	7·68

In the second set, which gives the highest ash, much iron was found to be present. It must be borne in mind that an increase in the weight of ash in the calcium pectate precipitate may be due either to the carrying down of suspended mineral matter or to the occlusion of organic substances such as the salts of organic acids. These latter if present may introduce considerable error, but if the juice is carefully filtered and if the precipitation is carried out in the presence of sufficient acid, it has been found that the difference can be reduced to a very small amount, which as is shown below may almost certainly be regarded as mainly of mineral origin.

It is significant to find that the impurities associated with pectin vary with different stages of pressing. This is illustrated by the following estimations:

Ash content of calcium pectate obtained from successive extractions of apple.

Number of pressings	Proportion of total Ca pectate present	Ash (calculated as percentage Ca)
1–10	79·5	7·62
10–20	14·0	9·20
20–30	6·5	9·70

In other cases final pressings were found to contain as much as 10–12 % of ash calculated as calcium. There seems much reason to think that this excess of ash is due to colloidal mineral matter, possibly associated with the protoplasm, which can only be pressed out when the cell is completely disintegrated. This, together with a variable amount of colloidal iron, appears to constitute the principal source of the impurities carried down with the calcium pectate precipitated from apple juice. The mineral nature of this impurity cannot be completely demonstrated without elementary analysis, but there seems to be very little doubt that it is largely, if not entirely, composed of inorganic material, in which case the error introduced into the weight of the calcium pectate precipitate will be very small.

It may accordingly be stated as a general rule that pectin should be estimated on material obtained by exhaustive extraction, but that if ash

determinations are made in order to ascertain whether or not the material affords calcium pectate in a state of comparative purity the later pressings should be rejected.

A few words may also be added here as to the precautions which it is advisable to take if satisfactory ash determinations are to be carried out. It must be remembered that pectin absorbs and carries down other substances very readily, and that these—once occluded—are removed only with very great difficulty.

Thus if calcium carbonate is contained in the precipitated gel, it is practically impossible to remove it by subsequent treatment with acid. Pectin should therefore be precipitated in dilute solution and in the presence of considerable excess of acid. The excess of $CaCl_2$ should not be greater than is sufficient for satisfactory coagulation, and at each stage of the process sufficient time should be allowed for the reagents to penetrate the large particles of the pectin sol.

The extraction of soluble pectin from apples.

A considerable amount of investigation has been necessary in order to work out a practicable method for the complete extraction of the soluble pectin. After the first few extractions have been carried out, further extraction gives a very dilute solution, and it is easy to imagine that the whole of the pectin has been extracted at this stage. It has been found, however, that a large proportion—sometimes more than 50%—comes out at so great a dilution that no precipitate is obtained from the solution even after prolonged standing with a large excess of alcohol.

The process is of necessity laborious. The following has been found the best method of procedure: 50 g. of finely minced apple are frozen in an efficient freezing mixture, and maintained at a low temperature for a considerable number of hours to ensure that the pulp is completely killed. The pulp is then warmed to the temperature of the air, and pressed through a cloth in a small hand press. After this the residue, which should be as dry as possible, is ground with fine purified sand in a mortar, and repeatedly washed with cold water and pressed. The residue should be well mixed after each addition of water. Sixty to eighty extractions are usually required, and the bulk of extract obtained is about two litres. Warm water should not be used, as this has been found to increase the amount of pectin obtained—presumably by promoting hydrolysis.

After extraction the liquid is boiled to destroy the action of pectase, and before estimations are carried out it is filtered, first through muslin and finally through a fluted filter paper. An aliquot part, containing a suitable quantity of pectin, is then taken for estimation. The amount varies from about one-half in the case of rather immature apples to one-tenth in the case of apples juicy or over-ripe.

The following estimations have been carried out on equal quantities of

apple previously finely minced and mixed to as uniform a condition as possible. They show the order of accuracy with which the process of extraction can be carried out.

Weight of calcium pectate	
In 1/10 of extract of 50 g. of apples	From 100 g. of apples
0·0257	0·514
0·0246	0·492
0·0271	0·542
0·0252	0·504
0·0245	0·490
Mean = 0·0256	Mean = 0·508

[*Note* added Feb. 17, 1922.—Other series of results have been obtained which show similar differences. These differences appear to be due rather to the difficulty of mixing the material uniformly than to incomplete extraction. Thus in the following series, in which pressings were collected in successive fractions, there is no indication that later fractions tend to contain more pectin where the first fraction is relatively low.

Weight of Ca pectate from 50 g. of apples			
Main portion	2nd fraction	3rd fraction	Total
0·339	0·0050	—	0·344
·345	·0100	0·0060	·361
·305	·0085	·0030	·3165
·328	·0070	—	·3345
·325	·0040	—	·329]

Summary.

The precipitation of pectin as calcium pectate is described. It is shown that by a careful adjustment of conditions this can be used as an accurate method of analysis, and that the precipitate corresponds in composition closely to the empirical formula $C_{17}H_{22}O_{16}Ca$. A method is given for the extraction of soluble pectin from apples, and the results of a number of similar estimations are compared.

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XIII. ON THE OCCURRENCE OF MANGANESE IN THE TUBE AND TISSUES OF *MESOCHAETOPTERUS TAYLORI*, POTTS, AND IN THE TUBE OF *CHAETOPTERUS VARIOPEDATUS*, RENIER.

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A number of marine annelids excrete a mucous substance which hardens to form a tube in which they live. Many of these tubes are partially soluble in cold dilute alkaline solutions.

In the course of an attempt to determine the nature of the material thus dissolved from the tube of an annelid which occurs commonly on the British Columbia coast, *Mesochaetopterus Taylori*, it was found that the substance precipitated on acidifying the alkaline extract yielded a bright blue ash on burning. Qualitative tests showed that this was due to manganese and led to the examination of the tube material and the tissues of the animal for that element. Preliminary quantitative tests indicated that it was present in both cases in unexpectedly large amounts and more careful determinations seemed warranted.

Traces of manganese are known to occur very generally in animal tissues and Bradley [1910] has shown that the element is normally present in considerable amounts in the tissues of fresh-water mussels. It was also observed by that author that the nacre of the shells of both living and fossil Unionidae is rich in manganese and this, so far as the writer is aware, is the only case of its occurrence in quantity in an animal secretion at all comparable with the tube of a worm.

All the more recent methods for the determination of manganese in organic material depend upon the extraction of the manganese salt from the ash and its oxidation to permanganic acid, which is estimated colorimetrically or by titration with arsenious acid or an arsenite. Reiman and Minot [1920] give an excellent summary of the literature of the subject and describe a method for determining the very small amounts of manganese occurring in human blood and tissues. In the present case the amount of manganese present was comparatively so large that the somewhat elaborate method of ashing the material and leaching the ash described by these authors was unnecessary. In working with the tube material it was, indeed, not found necessary to use

any oxidising agent to complete the destruction of the organic matter as is recommended by Bradley. Direct burning at a moderate temperature produced an ash, which, excepting some sand, was completely soluble in 35 % nitric acid, to which a few drops of hydrogen peroxide were added. Subsequent fusion of the undissolved residue with potassium nitrate rendered no more manganese soluble. In the case of the tissues the addition of a small quantity of potassium nitrate was found necessary. With this modification and substitution of a solution of sodium arsenite for that of arsenious acid for titration of the permanganic acid, the method used has been substantially that of Bradley. The details of procedure have been as follows:

0·2 to 1·0 g. of material is weighed into a procelain crucible. The bulk of the organic matter is burnt off at a low temperature. Potassium nitrate is then cautiously added if necessary. The crucible is then gradually brought to a bright red heat and maintained there for about half-an-hour. When cool 2 cc. of 35 % nitric acid and 5 drops of hydrogen peroxide are added to the contents of the crucible which is cautiously heated over a small flame. The acid is then diluted and decanted from the small amount of sand remaining undissolved, and the latter is repeatedly leached with small quantities of hot water until the combined leachings amount to about 50 cc. 0·5 cc. of 10 % silver nitrate is added to the solution which is then brought to the boiling point. The vessel is removed from the flame and 10 cc. of 20 % ammonium persulphate cautiously added. The permanganic acid colour develops immediately. The solution is then boiled gently for five minutes and cooled in running water. It is then titrated with a very dilute solution (not stronger than 0·1 g. per litre) of sodium arsenite which has been previously standardised against a solution of pure potassium permanganate reduced by sulphurous acid. An aliquot of this reduced permanganate solution is put through the foregoing oxidation procedure immediately before the sodium arsenite solution is standardised.

In application to aliquots of the standard permanganate solution evaporated to dryness this method gave results of a high degree of accuracy for quantities of manganese as small as 0·00001 g. It is important to boil the solution sufficiently long after addition of the ammonium persulphate to decompose the excess, otherwise too high readings are obtained. The presence of chlorides is stated by some authors to affect the accuracy of determinations of manganese depending upon the principle involved in this method. This has not proved to be the case in the work to be described. Traces of chloride were invariably indicated by the precipitation of silver chloride on adding the silver nitrate to the solution under analysis, but this dissolved on treating with ammonium persulphate and did not affect the determination. No appreciable difference in result was obtained whether the ash were heated with sulphuric acid, to drive off hydrochloric acid, before analysis or not. The figures recorded in Table I illustrate this point and indicate the degree of accuracy attained in duplicate samples.

Table I. *M. Taylori.*

Material	Weight taken g.	Manganese found %	Treatment
Tube	0·1592	0·0276	Ash not treated with sulphuric acid
,,	0·3426	0·0305	,, ,,
,,	0·2000	0·0300	Ash treated with sulphuric acid
,,	0·2176	0·0253	,, ,,
Posterior region	1·0690	0·0015	Ash not treated with sulphuric acid
,, ,,	0·7098	0·0015	Ash treated with sulphuric acid

Closer agreement than is indicated could not be expected owing to the difficulty of obtaining uniform samples. The material of the tube does not lend itself to very fine grinding and, as is subsequently shown (Table III), the different regions of the tube differ considerably in manganese content, so that such fluctuations as appear may easily be due to the regions being represented in varying proportion in the samples.

Determinations in individual tubes of Mesochaetopterus Taylori.

It is extremely difficult to dig up the tube of *M. Taylori* entire. It usually runs down more or less vertically through the fine sand for one to two feet and then takes abrupt turns in a more horizontal direction through the coarser material underneath. Almost invariably, therefore, unless very special care is taken, the tube is cut off short of the closed end. The tubes of which the analyses are given in Table II were the longest which could be selected from a large number dug, but only in the case of No. 1 was the terminal portion present. They were freed from adherent sand as far as possible, but varying quantities remained imbedded in the tube material. This was determined by weighing the residue after the ash had been completely extracted with nitric acid and water.

Table II. Tube of *M. Taylori.*

Serial number	Weight of material g.	Sand %	Manganese found mg.	Manganese in material %	Manganese in material – sand %
1	0·8402	45·9	0·297	0·0353	0·0654
2	0·3209	18·4	0·0715	0·0223	0·0273
3	0·2600	13·9	0·0132	0·0508	0·0589
4	0·2248	15·7	0·0385	0·0171	0·0203
5	0·1427	22·0	0·0495	0·0347	0·0445

The results show a considerable variation. It is noteworthy that although No. 3 gives the highest result on the entire material, this is in part due to its low content of sand and that, if calculation be made on the material minus sand, No. 1, in which the largest proportion of the basal end of the tube was present, gives the highest figure. This suggested that the basal end of the tube might be richer in manganese than the rest and that the variation in results was to some extent due to the varying proportion of this end of the tube represented in the material analysed.

Determinations in various regions of the tube of M. Taylori.

The tube can be easily divided transversely into three distinct regions corresponding roughly to the anterior, median and posterior portions of the animal. The uppermost, most of which projects above the surface of the sand bed, is composed of very thin papery material and is almost white. In the median region the wall is thicker and pale brown, whilst that of the posterior region is comparatively thick and frequently two or three layered. It is much darker in colour than the rest of the tube and often contains thickenings where the tube has been broken and repaired. Material from each of these regions was separated from a number of tubes and duplicate determinations made from the bulked samples. The results are given in Table III.

Table III. Regions of tube of *M. Taylori.*

Region	Weight of material g.	Ash %	Manganese found mg.	Manganese in material %	Manganese in material – ash %
Anterior	0·2520	49·2	0·011	0·0043	0·0086
,,	0·2126	46·4	0·011	0·0051	0·0096
Median	0·2746	39·9	0·0265	0·0096	0·0160
,,	0·3584	36·7	0·0385	0·0107	0·0169
Posterior	0·3426	26·7	0·1199	0·035	0·0475
,,	0·4250	27·2	0·1375	0·0326	0·0444

The duplicate samples agree as well as could be expected having regard to the difficulty of drawing uniform samples of material. The results show a marked difference in the three regions, the posterior being the highest and the anterior the lowest. The ash content varies in the inverse sense. The greater part of the ash was sand, but separate determinations of sand were not made in these cases. Calculated to the material minus ash there is still a very large difference between the manganese content of the three regions. There is no doubt this accounts in some measure for the irregularities shown in the determinations in individual tubes (Table II) since, as has been pointed out, these were not complete and it was in the proportion of material drawn from the posterior portion of the tube that they chiefly differed.

Determinations in tissues of M. Taylori.

The question whether the tissues of the worm itself contain manganese in notable quantity arises naturally out of its occurrence in the tube. The body of the worm is divided transversely into three distinct regions like the tube. For analysis these three regions were treated separately. They were removed from a large number of worms and kept in alcohol until hardened. The material was then dried, ground as finely as possible and duplicate samples from each region taken for analysis. The results are given in Table IV.

Table IV. Tissues of *M. Taylori*.

Region	Weight of material g.	Manganese found mg.	Manganese in material %
Anterior	0·7067	0·044	0·0062
,,	0·7653	0·0495	0·0065
Median	0·4985	0·077	0·015
,,	0·4493	0·0385	0·0086*
Posterior	1·069	0·016	0·0015
,,	0·7098	0·011	0·0016

* This result is certainly too low. No potassium nitrate was used in ashing and combustion was therefore not complete.

Manganese is present throughout the body of the worm, but in smaller proportion than in the tube. It is interesting to note that the median portion of the body contains most manganese since the chief glands which secrete the mucous material from which the tube is constructed are situated in this region.

Determinations in tube of Chaetopterus variopedatus.

C. variopedatus is the best known member of the family Chaetopteridae, but does not occur on our coast. A few tubes of this worm were obtained from Woods Hole, Massachusetts, for comparison with those of *M. Taylori* in respect of manganese content. The tube is a great deal wider in relation to the size of the inhabitant than in the case of the latter species and has both ends open. These both protrude above the surface of the muddy sand in which the animal lives. With the exception of these small and narrowed ends the material of the tube is very similar to the posterior portion of that of *M. Taylori*. It was found possible to divide the tube material longitudinally into two layers and it seemed of interest to determine manganese in the two layers separately. The inner one was smooth and clean and of a somewhat darker colour than the outer which was rough and heavily coated with sandy mud. This was cleaned as carefully as possible and the two lots of material dried separately. The results are given in Table V.

Table V. Tube of *C. variopedatus*.

	Weight of material g.	Ash %	Manganese found mg.	Manganese in material %	Manganese in material – ash %
Inner layer	0·3278	24·95	1·501	0·457	0·610
,, ,,	0·3042	28·27	1·314	0·431	0·602
Outer layer	0·2806	49·14	0·66	0·235	0·462
,, ,,	0·3021	45·81	0·676	0·224	0·410

The tube of *C. variopedatus* is thus more than ten times as rich in manganese as even the posterior portion of that of *M. Taylori*. The inner layer contains about twice as much as the outer one, but this is in part due to there being more sand in the latter. Expressed on ash-free material the ratio is about 3 : 2. This, coupled with the observation that manganese occurs in comparable quantity in the tissues of the allied species *M. Taylori*, suggests very strongly that the manganese in the tube is secreted by the worm and is

not due to the direct action of sand or sea water on it. It is probable, on the contrary, that these agencies tend to remove manganese from the outer layers of the tube since the sand lining the cavity from which a tube has been dug is invariably stained dark brown and looks precisely as if a rusty iron bar had been withdrawn. This rustiness is almost certainly due to oxides of iron and manganese resulting from the decomposition and wearing of the outside of the tube by sand and water.

In every case in which the test has been made iron has been found accompanying manganese in the ashes of the materials dealt with in this paper.

Examination of tubes of other worms for manganese.

The tubes of three other worms which occur commonly in this neighbourhood were examined for manganese; those of two *Sabellids* and a *Spiochaetopterus*. It was present in just sufficient quantity to be determined in the material from one of the sabellid tubes. No trace could be detected in that of the other sabellid or of the spiochaetopterus using quantities of material up to 0·75 g. The last case is particularly interesting because, not only is the animal nearly allied genetically to the chaetopterids in the tubes of which manganese occurs, but also it is always found in the sand beds living immediately alongside *M. Taylori*. We seem to have here a very marked instance of a physiological divergence in species taxonomically and ecologically similar.

Discussion.

As an outcome of the observations recorded the question of the source and function of manganese in the tubes of worms naturally arises. It is fairly clear, for reasons which have already been advanced, that the manganese arrives in the tube from the worm outwards and not from the sea water or sand inwards. Whilst it is conceivable that the worms concentrate manganese from the sea water direct it is not probable, because the quantity of the element in sea water is relatively very small indeed and the worms do not pass large quantities of water through their bodies, but strain their food material out of it. Enders [1909], who has carried out a detailed study of the life history and habits of *C. variopedatus*, holds that, in the case of that species, any sand which is suspended in the water taken by the worm into its tube is also discarded and that only diatoms and small animals are taken into the digestive tract. This does not, however, seem to be the case with *M. Taylori*, since a considerable quantity of sand was found in the posterior region of the animal. A sample of the diatoms which are normally to be found in the water covering the sand beds in which this worm lives, and which probably constitute a large part of its food, was obtained by dragging a fine net over beds of eel-grass growing in the immediate neighbourhood. The diatoms were separated by fractional sedimentation from the small animals and debris accompanying them, dried at 100° and ash and manganese determined. 60·8 % of

ash containing 0·007 % of manganese was found. The manganese was all readily extractable from the ash by 35 % nitric acid containing hydrogen peroxide. No further manganese could be detected after fusing the residual silica with sodium carbonate, so that it appears to be contained in the organic portion of the diatom. Here, therefore, we have an immediate source of manganese readily available to the worm. It is, however, not impossible that it is also derived from the sand which, in the case of *M. Taylori*, undoubtedly passes through the digestive tract in considerable quantity.

Manganese was determined in the sand in which the worms were living by sodium carbonate fusion followed by application of the method previously used, and 0·158 % found present. About a fifth of this was directly soluble in 35 % nitric acid and was therefore not in silicate combination. The sand passed through the animal therefore provides an ample source of manganese and its utilisation does not necessitate the assumption of any power of silicate digestion.

The presence of manganese in organisms has generally been associated with respiration. No evidence is at hand to show that it is to be connected with this activity in worms. It is difficult to see how its presence in the tube can have any connection with respiration, and, in the absence of any evidence to the contrary, it seems most rational to assume that its presence there is of no physiological significance, but is due to the worm excreting its superfluous manganese with the tube-building material, a theory which is supported by the observation of manganese in larger quantity in the lining than in the outer layers of the tube of *C. variopedatus*, and in the mucous secreting region of the body of *M. Taylori*, more than in the other regions. The presence of more manganese in the posterior than in the other portions of the tube of the latter species is also explained on this assumption since this is the oldest part of the tube and has been most frequently relined and repaired. Bradley [1910] has shown that the excreta of fresh-water mussels living on their normal manganiferous diet contain the element in about the same proportion as it occurs in their food, so that a state of manganese balance is maintained—only if they are deprived of manganiferous food is the metal definitely retained by the animal. It is likely that a similar state of affairs exists in the case of worms having manganiferous tubes, but that some of the superfluous manganese passes into the tube material. The deposition of manganese in the nacre of the shell of the Unionidae observed by Bradley is no doubt a parallel case.

Summary.

1. Manganese occurs throughout the tube of *M. Taylori*, but in much greater quantity in the posterior portion than in the other parts.

2. Manganese is present throughout the body of *M. Taylori*, but in smaller quantity than in the tube. The median region of the body, in which the chief mucous secreting glands are situated is richest in manganese.

3. Manganese occurs in far larger quantity in the tubes of *C. variopedatus* than in those of *M. Taylori*. The lining of the tube contains much more manganese than the outer layer.

4. The tubes of local species of *Sabellid* and *Spiochaetopterus* contain no manganese.

5. Manganese is probably present in worm tubes as a waste material excreted by the worm with its tube building substance. It may be derived either from diatomaceous food or from sand passing through the digestive tract with the food, or from both.

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XIV. MAMMARY SECRETION. III.

1. THE QUALITY AND QUANTITY OF DIETARY PROTEIN.
2. THE RELATION OF PROTEIN TO OTHER DIETARY CONSTITUENTS.

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Introductory and Historical.

In a previous paper [Hartwell, 1921, 2], it was shown that large quantities of protein in the diet of a lactating rat were detrimental to the young. The diet consisted of bread and protein in the proportion 15·0 g. bread to 5·0 g. protein. An obvious criticism was that such a diet is deficient in fat and vitamins, but it was pointed out that it was unlikely the bad effects were due to deficiency since the rat could bring up a healthy litter on a diet of white bread alone, a diet equally deficient.

It is generally held that the diet of a nursing mother should be rich in protein, because young growing animals need large amounts of protein in their diet. It is probable that if large quantities of protein are ingested, some other factor must be present in the diet to safeguard the young from such harmful effects as those previously noted. The object of the present investigation was, therefore:

1. To find out what proportion of protein can be fed to the mother without danger to the offspring.

2. To determine what other factor or factors should be included in the diet in order that the large quantity of protein may be effectively metabolised.

It is probable that not only should the diet be physiologically complete, but that a greater quantity of one or more constituents might be necessary when the intake of protein is high. For instance, when large quantities of fat are ingested, it is essential that carbohydrate should be included in the diet; otherwise the fat is not properly metabolised and acetone bodies are excreted.

It is also probable that the large amount of protein deemed necessary has been somewhat overestimated, and that less amounts would give equally good results provided that the diet was satisfactory in other respects.

That excess of protein in the mother's diet has detrimental effects on the young is evident from the experiments of other workers, but such results have been explained differently.

The experiments of McCollum and Simmonds [1918] are on almost identical lines with those described in this paper. These observers, however, were working with diets which were inadequate for growth of the young, and the main object of their research appears to be to show how far "the mother can serve as a protective agent in producing milk suitable for the nutrition of the young," when she herself is getting a deficient diet. A great difference between the work of McCollum and Simmonds and that of the research now being described is the duration of the experiments. From the former's curves it appears that the mother was left with the young for 40 days or more, which seems rather long, considering that a normal baby rat is perfectly capable of living alone from the 21st day. In one or two of their experiments the symptoms of the litter closely resemble those described in this series of experiments. In McCollum's experiment 738 the mother rat was fed on rolled oats alone, and certain symptoms were noted in the young. "On about the 20th day a few of the young would throw themselves about the cage, and scream during this performance. Several of the young went into coma and died."

In some experiments where the mother appeared to have a high tolerance for protein, the babies behaved in the way McCollum describes about the 20th–24th days; they had shown no symptoms at the earlier stage. Again in experiment 948 he says that "the young all died at a very early date. The intestines were filled with gas. We are unable to account for the high mortality of these young." The diet used in this case was rolled oats 82·0 and caseinogen 18·0, *i.e.* a greater proportion of protein than when the rats had rolled oats only. The young died at intervals from the 3rd to the 23rd day. Seven experiments were made.

The condition of the young certainly suggests a similar state to that found in this work [1921, 2], but only a few of the typical symptoms are recorded by McCollum and Simmonds. In their paper they state that the babies were weighed "frequently" and the curves are plotted at intervals of eight days. Unless daily weighings were made, and the babies carefully examined, it is highly probable that the spasms would be overlooked, since this stage is frequently of short duration, sometimes lasting only a few hours. The screaming fits exhibited by the older babies are much more obvious, the noise being distinctly heard in the next room.

It is possible that these results are due to a deficiency of some dietary factor, but this deficiency is only evident when the diet is too rich in protein and therefore it may be that they are directly due to the excess of protein. A similar diet, but with less protein, would not be deficient.

General condition of the animals.

Only normal healthy animals were used in these experiments. If any rat became ill the results were discarded. There was no trouble with scab, pneumonia, or epidemic diseases. McCollum [1920] says that the "palatability of the ration is the most important factor in animal nutrition," and it is a well-known fact that many animals will starve rather than eat food which is distasteful to them. The rats used in this work readily ate any of the mixtures offered to them, and in some cases consumed enormous quantities. For instance, many rats were taking as much as 40·0 g. bread (+ added protein and other constituents) on the ninth day of lactation. The initial ration of 15·0 g. bread etc. was usually adequate for the first two days, after which the animals' appetites increased rapidly. The cages were examined for any hidden food, the "food-hoarding" instinct being especially marked in rats.

The males and females were kept together until a few days before the birth of the litters, when the mothers were removed and placed in separate cages. After lactation the females were kept from the males for about two weeks to avoid over-strain of the maternal organism, and to give the mother a chance of recovery, should the special diet have upset her in any way. This method of procedure was adopted merely as a safeguard, because even when the litters died, the mothers appeared fit and well. Except during the actual period of lactation (and with one exception pointed out later) the males and females were fed on a diet of kitchen scraps, supplemented with small quantities of whole milk and white bread. This mixture proved a very good one and, as is shown later, the growth curves of the young animals fed on such a diet, are better than those given by Donaldson [1906] as normal for the white rat.

Some white rats, and some white and black were used. It is possible that the latter should be somewhat heavier, since Donaldson states that black rats are heavier than white ones, yet there appeared practically no difference in size and weight.

Methods.

The technique employed was the same as that previously described [Hartwell, 1921, 1].

The litters, as before, were reduced to six. In a few cases less than six were born, and this is noted in the text and on the curves. The mothers and babies were weighed daily and the weights recorded graphically. In the "foster" experiments to be described later, the number in the litter varies, but is always stated.

The foodstuffs used. (*a*) Pure caseinogen was not obtainable when these experiments were in progress, and a commercial form, "food casein,"[1] was therefore used. Experiments showed that the results obtained with "food casein" were exactly similar to those previously found with pure caseinogen.

[1] I am indebted to Casein, Ltd., for supplying me with the composition of "food casein."

The "food casein" was 83 % pure, and the amounts given were calculated in terms of pure protein.

(*b*) Pure egg albumin (as supplied by Baird and Tatlock) was used. It was very finely ground, in order to obtain an even mixture of the foodstuffs fed.

(*c*) A commercial preparation of edestin was used. It was tested at the beginning of the experiments and found to contain only slight amounts of impurities. A new sample was tested lately and found to contain cane sugar. Every sample was not analysed, and as that obtained lately was less pure, it is possible that the results obtained with 1 g. and 2 g. edestin are due to the fact that smaller amounts of protein were actually fed.

To find what amount of protein is really excess, it would be essential to work with absolutely pure foodstuffs.

(*d*) The lactose was pure, as supplied by Baird and Tatlock.

(*e*) The salt mixture was that recommended by Mottram, and is a modification of the original one used by Hopkins.

Sodium chloride NaCl	46·25 g.
Magnesium sulphate (anhyd.) $MgSO_4$ ...	71·20
Acid sod. phosphate NaH_2PO_4, H_2O	92·68
Di. pot. phosphate K_2HPO_4	254·60
Calcium tetrahydrogen phos. $CaH_4(PO_4)_2H_2O$	144·20
Calcium lactate Ca $(C_3H_5O_3)_2$ $5H_2O$	347·00
Ferric citrate Fe $(C_7H_6O_7)$ $1\frac{1}{2}$ H_2O	31·52
Sodium fluoride NaF	·55
Manganese sulphate $MnSO_4$ $5H_2O$	2·00
Potassium iodide KI	10·00
Total...	1000·00

Water, *ad lib*, was always given unless the rats were having large quantities of milk.

1. The quality and quantity of dietary protein.

In some recent experiments [Hartwell, 1921, 2] the initial diet consisted of 15·0 g. bread to 5·0 g. protein, both being increased proportionately. Only a very few babies survived and those which lived were not normal, therefore in these experiments less protein was given to the mother, in order to see if the litter would then survive. Small quantities of commercial yeast extract, marmite (about 0·2 g. per day) were added to the diet to make it more palatable. It has been found that small amounts make no difference to the final results and the animals ate such a mixture more readily.

Three experiments were made with each protein. This involved 9 mothers and 54 young. The diet was not physiologically complete except in two of the experiments, when 0·5 g. butter, 0·7 cc. lemon juice and 0·7 g. salt were added to the mixture; this made no difference to the final results.

(i) *Proportion of* 15 *g. bread to* 1 *g. protein.*

(*a*) Caseinogen. Fig. 1 *a*.

Exps. 160 and 173. 15·0 g. bread, 1·0 g. caseinogen, marmite.

Exp. 175. 15·0 g. bread, 1·0 g. caseinogen, marmite, butter, lemon juice and salt mixture.

Litter 160 were perfectly normal in every respect. One was weakly and died three days after weaning, but no typical symptoms (previously described [1921, 2]) were exhibited.

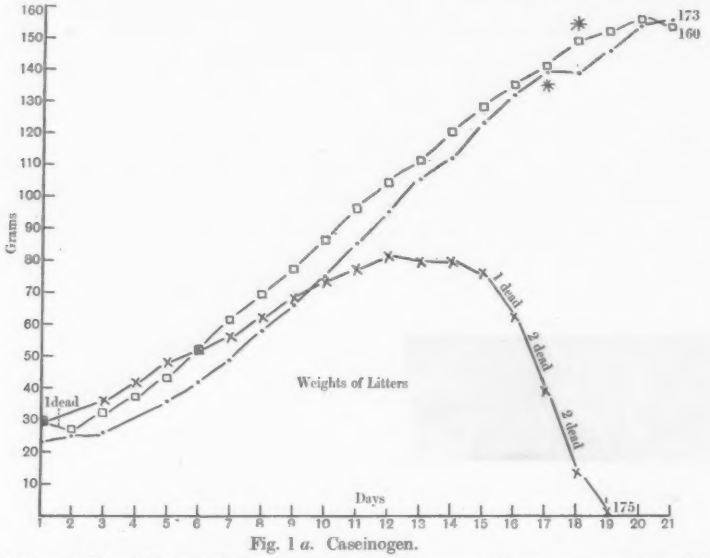

Fig. 1 *a*. Caseinogen.

Litter 173 exhibited slight symptoms. However they recovered rapidly, the mother was removed on the 21st day, when all the babies appeared normal. They all survived weaning. On the 21st day, the average weight of babies 160 was 30·6 g. and that of litter 173 was 26·0 g. From the normal curve according to Donaldson [1906] the weight at 21 days is about 21·0 g. Hewer [1914] states that she used for subsequent feeding experiments any young rats which weighed at least 23·0 g. at weaning, so it would appear that both litters described above were well above average weight, in spite of the fact that slight symptoms developed in 173. It is possible that "breed" and number in the litter are factors influencing the actual weight of the babies. This is discussed later in the paper.

Litter 175 showed bad symptoms and were all dead by the 19th day.

The mothers all remained well. They were not full grown at the beginning of the experiment and put on a considerable amount of weight.

(*b*) Edestin.

Exps. 141 and 195. 15·0 g. bread, 5·0 g. edestin, marmite.

Exp. 163. 15·0 g. bread, 5·0 g. edestin, marmite, butter, lemon juice and salt mixture.

All litters were normal in every respect, none of the typical symptoms were noticed in any one baby, not even excitability. The rate of growth in the middle part of lactation was extraordinarily good and apparently maximal (*i.e.* as good as when the mother is fed on bread and milk, a diet previously shown to give the best results).

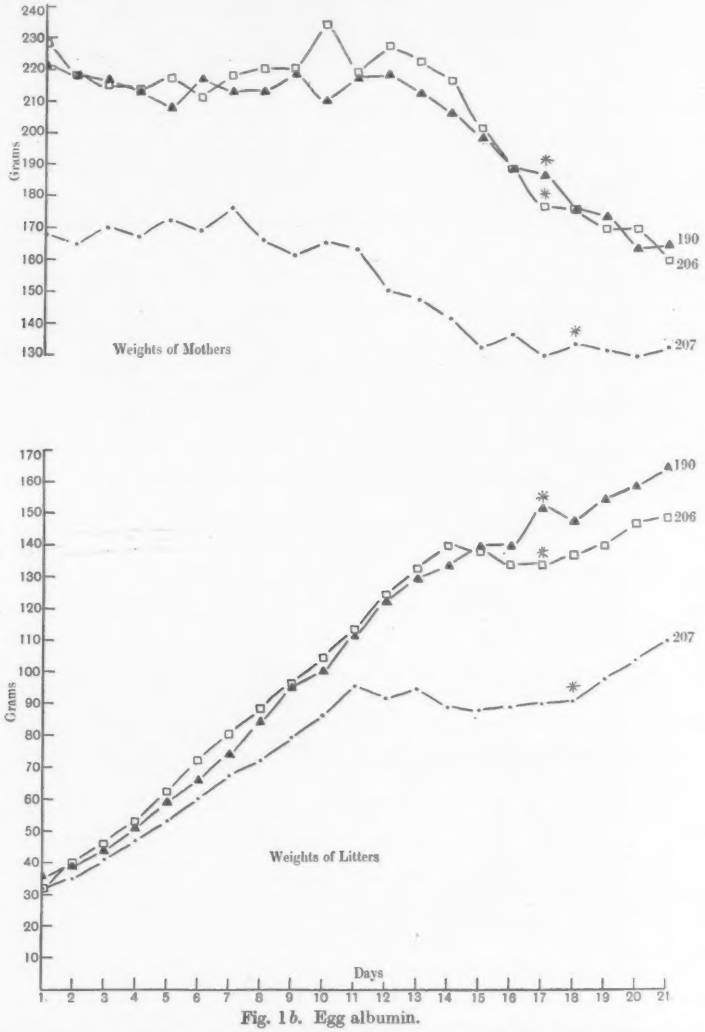

Fig. 1*b*. Egg albumin.

The mothers, however, showed a slight loss of weight during the first two weeks and this loss became very marked during the last week of lactation.

This seems rather extraordinary because during the last part the young were able to eat for themselves, and were not entirely dependent upon the mother.

(*c*) Egg albumin. Fig. 1 *b*.

Exps. 190, 206, 207. 15·0 g. bread, 1 g. egg albumin, marmite.

In these experiments no litter was quite normal, but the mothers were removed on the 21st day and all the babies were successfully weaned.

Litter 190 showed only slight spasms, and recovered rapidly. Two days after the mother was removed they were normal, but small and somewhat thin.

Litter 206 were very similar to 190, but their condition was a little worse and they were not normal till a week after weaning. It will be seen from Fig. 1 *b* that this litter lost weight for three days before they began to eat for themselves.

Litter 207 showed worse symptoms than 190 and 206 but eventually all the babies recovered.

At first the mothers maintained their weights or showed a slight loss, but from the 11th or 12th day they lost weight considerably. In ten days two of them lost approximately one-quarter of their body-weight and the third lost about one-fifth body-weight. The significance of this is discussed later (p. 86). Thus when 1·0 g. protein to 15·0 g. bread was fed to the mothers, slight symptoms developed in the litters, except with edestin. This is probably due to the fact that commercial edestin was used and, therefore, the mothers were receiving less amounts than with the other proteins.

(ii) *Proportion of* 15·0 *g. bread to* 2·0 *g. protein.*

(*a*) Caseinogen. Fig. 2.

Exps. 161 and 198. 15·0 g. bread, 2·0 g. caseinogen, marmite.

Exp. 176. 15·0 g. bread, 2·0 g. caseinogen, marmite, butter, lemon juice and salt mixture.

Litter 198 were normal in all respects except that they were somewhat excitable and, as will be seen from the curves, gained only small amounts the two days before they were able to eat for themselves.

Litter 161 showed the typical "excess protein" symptoms, but two lived till the 21st day and survived weaning, although they were not normal till two weeks after.

Litter 176 were in a worse state than 161 and were all dead by the 18th day.

The mothers 161 and 176 put on weight, whilst 198 lost weight. In this connection it is interesting to note that 198 litter were normal, but 161 and 176 suffered badly. This is in agreement with a suggestion previously made that there is some correlation between the weights of the mother and babies.

(*b*) Edestin.

Exps. 143 and 215. 15·0 g. bread, 2·0 g. edestin, marmite.

Exp. 197. 15·0 g. bread, 2·0 g. edestin, marmite, butter, lemon juice, salt mixture.

Litter 215 were normal except that they developed slight "toe walking" towards the end of lactation. They recovered rapidly and were successfully weaned. Two of the babies died the 2nd day and, therefore, the mother had

only four to suckle. Since the average weight of these (see table on p. 87) is so much better than that of the two other litters, it is possible that it can be accounted for in this way. The fact that this rat may have had a greater tolerance for protein is also a factor to be considered.

Litter 197 exhibited all the usual symptoms to some extent, but were never very bad. They soon recovered and were successfully weaned.

Litter 143 developed very bad spasms accompanied by the usual symptoms. One died the 19th day and the others were all dead two days after the mother was removed.

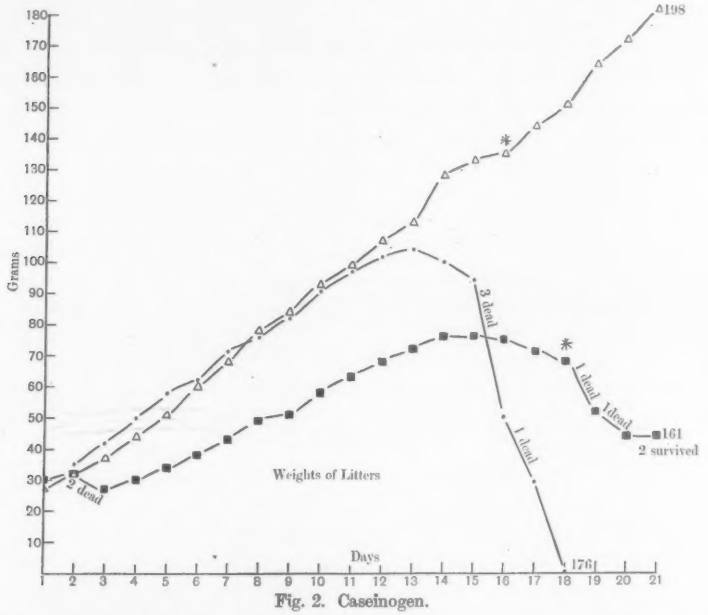

Fig. 2. Caseinogen.

The mothers' curves are somewhat irregular, but on the whole show a maintenance of weight.

(*c*) Egg albumin.

Exps. 189, 208, 209. 15·0 g. bread, 2·0 g. egg albumin, marmite.

All the litters survived and none showed any spasms.

On the other hand none of the babies was quite normal. Litter 189 did best, but were somewhat excitable. Litter 208 were very excitable, and showed "toe walking."

Litter 209 had similar symptoms to a greater degree and were cold and very weak.

The mothers appeared well, but, as in the case of those which received 1·0 g. egg albumin, they lost weight rapidly.

Discussion of Results.

The average weights of the young rats at 21 days are given below:

Caseinogen	Edestin	Egg albumin
1·0 g. protein:		
30·6	31·16	27·33
26·0	31·4	24·66
All died	31·16	18·16
2·0 g. protein:		
30·33	33·0 (after 2nd day only four)	29·16
22·0 (only two survived)	24·33	23·5
All died	20·40	19·33

(i) *Irregular growth of the young.*

It has been previously pointed out [Hartwell, 1921, 1] that on any given diet for the mothers, similar growth curves could be obtained for the litters, and that such curves could be regarded as characteristic for that specific diet. This statement now needs some modification.

The experiments described in this paper show that on any given diet the growth curves of the sucklings are by no means identical. It seems probable that when sufficient protein is given to produce bad symptoms in the young, then the above statement no longer holds good. This is well illustrated by the figures above. The average weights of a 21 day rat when the mother received 1·0 g. edestin are practically constant. These litters were normal in every way, so it appears that this amount of protein was not excessive. It is possible that less than 1 g. protein was given in these experiments, since recent samples of the commercial protein have been shown to contain cane sugar as impurity.

In all the other experiments, varying degrees of abnormality were produced and the weights are by no means constant.

This point is again illustrated by experiments to be described later on in this paper.

Thus with sufficient protein in the mother's diet to produce bad symptoms in the young, the individual metabolism of the mother rat seems to play an important part.

The mother's initial weight is not a factor to be considered, since on any given diet the babies of a heavy rat may die while those of a lighter rat may survive, or *vice versa*.

(ii) *Correlation between weights of mothers and offspring.*

It was previously suggested that there was some correlation between the weight of the mother and condition of the offspring, and that if the mother lost weight she might be able to spare the babies some of the symptoms. This suggestion is confirmed by these experiments.

In Fig. 2 litter 198 have a fair growth curve, and showed no symptoms. The mother lost weight. Litters 176 and 161, on the other hand, exhibited

typical symptoms and only two of the 12 survived. These mothers put on weight.

In both sets of experiments (*i.e.* 1·0 g. and 2·0 g. protein) it is very obvious that the mothers fed on egg albumin lost weight considerably.

Since albumin is a less good protein than either edestin or caseinogen, it may be that the mother is supplying the necessary amino-acid constituents from her own tissues.

Even 5·0 g. egg albumin to 15·0 g. bread results in some loss in weight of the mother, in a deficient diet [Hartwell, 1921, 2], but practically no loss is found when that proportion is given in a physiologically complete diet (experiments described later).

From the following figures it can be seen that, in some cases, the mother rat lost as much as one-third of her body weight; moreover this loss was very rapid and occupied only ten days.

	Exps.	Weight at beginning of lactation. g.	Weight at end. g.	Loss g.
1·0 g. egg albumin:				
	190	221	164	57
	206	228	159	69
	207	168	142	26
2·0 g. egg albumin:				
	189	247	165	82
	208	214	174	40
	209	149	128	21

Rat 207 lost less than 190 and 206 and her litter were in a worse condition and gained less weight than those of 206 and 190.

Again, in the other series, rat 209 lost less and her litter were the worst.

(iii) *The amount of protein constituting excess.*

It is not possible to give a definite statement as to the proportion of protein which should be fed to a lactating animal, since with different proteins, a different amount will constitute excess. For instance, good growth curves were obtained when the mother was fed with 15·0 g. bread and 1·0 g. edestin, but in the three litters whose mothers had 1 g. egg albumin, there were no normal babies; with caseinogen, one litter was normal, one had slight symptoms and the babies of the third litter all developed bad symptoms and died. As previously explained (p. 81) there is a possibility of impurity in the edestin and hence the animals might be getting less than 1·0 g. of protein, which might account for the slightly better results obtained with the edestin.

It is probable that the amount of protein constituting excess will vary with the presence or absence of other dietary constituents, and that such constituents must be considered *quantitatively* in relation to the protein. This point will be dealt with later in the paper. With caseinogen and edestin, a diet which is complete physiologically from a qualitative point of view gives just as bad results as one not physiologically complete. For example rat 176 received butter, lemon juice and salt mixture in addition to bread, caseinogen and marmite, thus completing the diet qualitatively, yet her litter were worse

than those of 198 and 161, whose mother had only bread, caseinogen and marmite.

(iv) *The quality of the protein.*

Certain typical symptoms, already described, were characteristic of the litters, irrespective of the protein fed to the mothers. With egg albumin, however, some extra symptoms were noticed. The weakness appeared to come on at an earlier stage, the babies were thinner and were very cold to the touch. This coldness was observed at an early age and was frequently the first indication that something was wrong. It was evident even when the symptoms were slight and the babies recovered. It is possible that the thinness of the babies is due to less good milk being produced by the mother. It is suggested that egg albumin is deficient or poor in certain amino-acids which are essential for the production of milk of adequate nutritive value. To prove such a point it would be necessary to feed with pure amino-acids.

2. THE RELATION OF PROTEIN TO OTHER DIETARY CONSTITUENTS.

(i) *The effect of large quantities of protein in a physiologically complete diet.*

Hartwell [1921, 2] showed that an initial diet of 15·0 g. bread and 5·0 g. protein fed to nursing rats was detrimental to the young. It was, however, pointed out that such a diet was deficient physiologically, yet it was unlikely the bad symptoms were due to such deficiencies, since the rat can bring up a healthy litter when fed on white bread alone [Hartwell, 1921, 1], a diet also lacking in fat, vitamins and salts.

In these experiments the proportion of protein to bread was kept the same as that used in the previous investigation (*i.e.* 15·0 g. bread to 5·0 g. protein), but 0·5 g. butter (fat and vitamin *A*), 0·2 g. marmite (vitamin *B*), 0·7 cc. lemon juice (vitamin *C*) and 0·7 g. salt mixture (composition given on p. 81) were added to complete the diet.

Three proteins were tried, caseinogen, edestin, and egg albumin and in each case two series of experiments were done.

1. The diet started as soon as possible after the birth of the litter; never more than 24 hours.

2. Diet started before the birth of the litter, and at different stages of pregnancy.

The bread and protein were increased proportionately as required, but the other constituents remained constant.

(*a*) Caseinogen. Fig. 3.

Exps. 113, 194, 171. Diet started *at* birth of litter.
Exp. 126. Diet started 10 days *before* birth of litter.
" 133. " 13 " "
" 134. " 16 " "
" 166. " 27 " "

The gestation period in the rat is given by some observers as 21 and by others as 23 days, so in the second series of experiments the rats were getting the excess protein diet for at least half the gestation period. Rat 166 received this diet during the whole of gestation. In this animal, either the gestation period was longer than normal, or else fertilisation was delayed, because she was separated from the males when the special feeding was started and the litter were not born for 27 days.

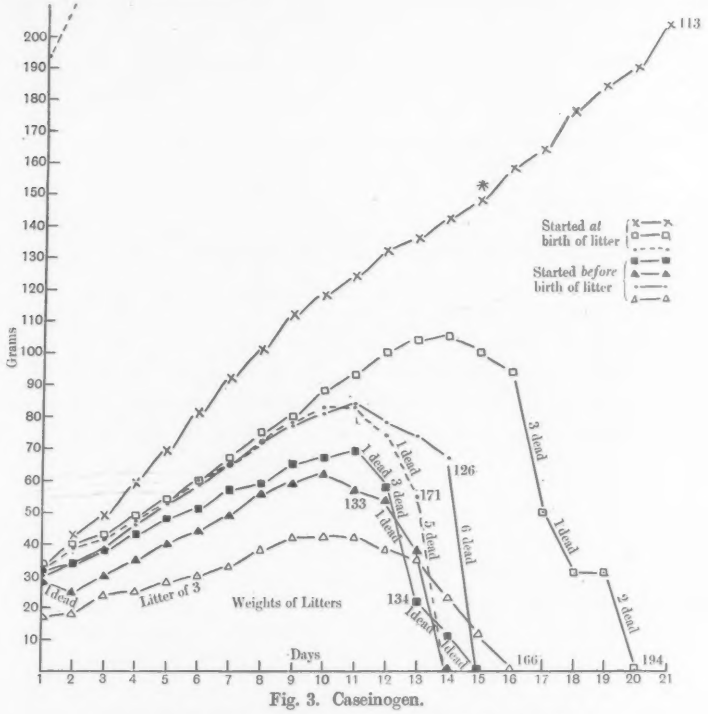

Fig. 3. Caseinogen.

A. *Feeding started after the birth of the litter.*

Litter 113 became excitable on the 9th day and from that time onwards showed signs of being hungry.

No bad symptoms became evident until the 19th day when four of the litter had very bad fits, screamed, rushed round their cage, bit each other and hung on to the cage with firmly fixed jaws. These fits lasted from one to five minutes. Afterwards the babies were very exhausted and would sprawl on the floor of the cage. At this stage the mother frequently removed them to the nest and covered them up. The mother was taken away on the 21st day and all the litter survived, in spite of the fact that bad screaming fits were again noticed on the 22nd day.

Litters 194 and 171 gave the typical "excess protein" curves and all died. The condition of the alimentary canals of the babies was exactly the same as previously described [Hartwell, 1921, 2].

B. *Feeding started before the birth of the litter.*

The actual duration of the feeding before birth seems to be an unimportant factor, since all four curves are similar and the babies were all dead either the 14th, 15th, or 16th day, irrespective of when the feeding was started. In some experiments to be described later (p. 92) the mothers were fed on the caseinogen diet from the time they themselves were weaned and had, therefore, been eating this mixture for 70 days before the birth of the litter.

(*b*) Edestin.

Exps. 177, 112. Feeding started *at* birth of litter.
Exp. 124. Feeding started 2 days *before* birth of litter.
„ 146. „ 11 „ „
„ 132. „ 21 „ „

A. *Feeding started at birth of litter.*

Litter 177 showed quite good growth up to the 11th day, after which they only gained small amounts. They exhibited all the typical symptoms, though not so markedly as usual. They ate for themselves on the 16th day and were all successfully weaned. They were not normal for about ten days.

Litter 112 grew much more rapidly than did 177, but they developed bad symptoms earlier and were much worse in every way. Only one survived and it was weakly for a month after being taken from its mother.

B. *Feeding started after the birth of the litters.*

In these experiments the time of feeding before birth bears a definite and inverse relation to the time the babies lived, but this does not apply to the litters whose mothers were fed with caseinogen and egg albumin.

There is no reason to describe the experiments separately, or in detail, as the results are similar to those already described for caseinogen.

(*c*) Egg albumin.

Exps. 111, 193, 221. Feeding started *at* birth of litter.
Exp. 125. Feeding started 4 days *before* birth of litter.
„ 129. „ 11 „ „
„ 145. „ 9 „ „
„ 130. „ 15 „ „

A. *Feeding started at birth.*

Litter 111 had the best growth curve in this series, but bad spasms were seen on the 13th day and after this the usual bad symptoms were marked.

Only one of the litter survived and it was very weak and backward in every way. It did not eat for itself till the 21st day and therefore the mother was not removed until the 24th day. After this the young rat was weakly for some time, but survived.

Litter 193 were all dead the 17th day, having suffered from all typical symptoms, though not to a marked degree. It has been noticed that if extreme weakness comes on at an early stage, the acute spasms are frequently absent.

Litter 221 suffered badly. They showed spasms and all typical symptoms. They were all dead the 16th day.

B. *Feeding started before birth of litter.*

These results again agree with those obtained with edestin and caseinogen.

With all three proteins the mothers were very fit and in most cases put on weight.

From these experiments one would conclude that feeding the mother rat with protein in the proportion 15·0 g. bread to 5·0 g. protein, even in a physiologically complete diet is harmful to the litter. If the feeding is started before the birth of the litter, then no babies survive; also it appears to make very little difference if the feeding is started a few days before the birth or maintained during the greater part of gestation.

Failure in rearing the babies is of much more frequent occurrence when the mother is given an excess of protein in the diet (*at* birth of litter) than when she is fed on other mixtures. It is very easy to see if the young rats have been fed, and with an excess protein diet it was not infrequently found that the whole litter died in the first two or three days, and had hardly had any food.

The symptoms develop at approximately the same time and this seems to suggest a correlation with some stage of development of the young. This possibility is supported by the fact that in a foster litter the symptoms also develop about the same time (experiment to be described later).

If the feeding is started *after* the birth of the litter, some babies may survive, but they are never normal; the majority die.

The results with egg albumin are less good than those with caseinogen and edestin, but this as already suggested is probably due to the different composition of the proteins. Other differences will be commented on later (p. 98).

The fact that some litters do better than others (*e.g.* litter 113 show a better growth curve than any other on the same diet) is possibly due to the greater tolerance of certain rats for high proportions of protein. In this connection it is also probable that the loss of weight of the mother is a factor to be considered; rat 113 lost weight, while other rats on that diet gained and their litters died.

Thus the bad effects on the litters are obtained even when the diet is physiologically complete. It should be pointed out that the amounts of butter (fat and vitamin *A*), marmite (vitamin *B*), lemon juice (vitamin *C*) and salt mixture (inorganic constituents of diet) used were adequate for a normal diet

(see experiment described below, when weanlings were fed on a similar mixture).

The mothers remained well during the experiments and when the diet was started before the birth of the litter, the period of feeding lasted as long as five weeks.

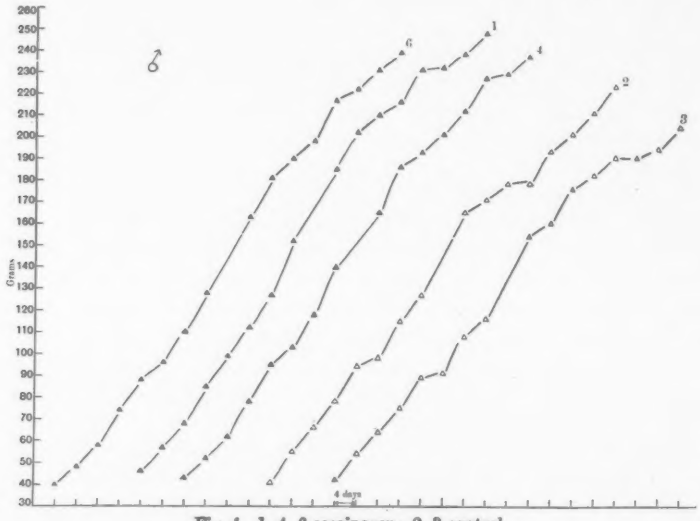

Fig. 4. 1, 4, 6 caseinogen; 2, 3 control.

Growth of young rats on a diet similar to that fed to the mother. Fig. 4.

One criticism which might be offered is that although the mother rats appeared well, they might not have remained so had the feeding been continued longer. To test the adequacy of this diet, young rats were given a similar mixture. Two normal healthy litters were chosen and fed on bread and milk for two days after weaning. Then half of each litter was put on this diet, the protein used was caseinogen, and the other halves of the litters were used as controls, being fed on kitchen scraps supplemented with small quantities of bread and milk.

The young rats were weighed separately every day at first and later every four days. The increase in weight of the males is shown in Fig. 4.

The caseinogen-fed males did better than the controls, but the females grew equally on both diets.

The experiment lasted 90 days, which should prove a suitable length of time for testing the diet.

The protein-fed animals grew rapidly and were very fit, their coats were thick and silky and decidedly better than that of the average animal. The females became pregnant and the litters were born at about 13 weeks, which

is recognised as the normal time for the appearance of the first litter. In the controls the first litter appeared about two weeks later. The three caseinogen-fed rats produced healthy litters, which compared very favourably both in size and weight with the average first litter.

The litters were not reared. Their growth curves were typically those described previously when the mothers received excess protein in the diet.

The weights of the animals at 70 and 90 days are compared with those of Donaldson [1906] and Osborne and Mendel [1911].

The average weights given by Osborne and Mendel are slightly less than those of Donaldson. The authors suggest that this is due to differences of "breed."

The rats used in these experiments were black and white and as before suggested they may be of a bigger breed. In other experiments both white, and black and white animals were used, and practically no differences in weight were noticed. The only difference was that the white rats do not make such good mothers and, therefore, for the later experiments, they were rarely used.

	Weight of rat at 70 days Average weight		Weight at 90 days Average weight	
	Male	Female	Males	
	g.	g.	g.	
Donaldson	107	100	150	
Osborne and Mendel	120	100	135	
Caseinogen-fed	(3) 198, 201, 216	(3) 114, 143, 165	(3) 231, 242, 258	Females not given because they were pregnant
Controls. Fed on kitchen scraps and small amount bread and milk	(2) 178, 182	(3) 103, 158, 162	(2) 204, 223	

This experiment proves the diet to be adequate in all respects except during lactation. It is extraordinary that a diet, so obviously good for a growing animal, suitable for fertilisation and production of offspring, should cause such disastrous effects on the suckling babes.

Experiments with foster-mothers.

It was suggested on p. 91 that the bad symptoms in the babies were in some way connected with development, since the symptoms usually came on at about the same time. It does not seem at all feasible to suppose that the milk contained toxic substances at a definite time, and then lost these properties again. In support of this view it was found that if a rat could be induced to suckle more babies after her own had all died and the excess protein feeding continued, then the new litter gained for a time though not to any great extent, but finally died. The growth curve for the young is very similar to that obtained with the rat's own litter, except that the increase of weight of the babies is less than with the original litter. (This fact applies also to a good diet, see experiment 107.)

Several "foster" experiments were made. Rat 152 was fed on bread

edestin, butter, marmite, lemon juice and salt mixture (the same diet as previously used) while nursing her own babies (experiment 112), and the diet continued with the foster litter, five in number. The foster babies gained weight until the 9th day, then ceased to gain, finally lost weight and died. No spasms were noticed, but weakness was a prominent feature. It was frequently found that slower-growing babies developed weakness earlier and showed only slight spasms, or none at all; however, this was not always the case. The mother, 152, lost weight considerably, but in experiment 112 she had gained a good deal. At the beginning of experiment 112 she weighed 212 g. and at the end of experiment 152 she was 205 g. In this instance loss of weight of the mother did not spare the litter, but the mother's loss is hardly comparable to that of other experiments, because in this case the rat had just put on what she now lost.

Rat 107 brought up foster babies when she was having a diet of bread and milk. Previously she was fed on a diet of egg albumin and bread, and all her own litter had died, with typical symptoms. These two experiments are representative of others, and it is inferred that after her own babies have died as a result of too much protein in her diet, the rat is capable of bringing up another litter. If a good diet is given in the second experiment, the babies are normal but small. They do not develop so rapidly as a rat's own babies, and are not able to eat for themselves until about the 21st day. If the excess protein diet is continued, the babies gain small amounts for a time, but eventually die, having shown the usual symptoms, except that they do not suffer from spasms.

In the experiments in which the mothers received excess protein, the foster babies did not develop symptoms until a few days later than the original babies. It has been pointed out that on a good diet the foster babies do not eat till later. This is in agreement with the suggestion that the development of the young is a factor to be considered.

Quite a number of rats were unable to bring up a foster litter after their own had died as a result of too much protein in the diet. The rats took to the babies and mothered them, but were apparently unable to feed them. The babies lived from two to four days, during which time they lost weight. They did not appear to have been fed, although the rat took care of them in the nest. These babies were rarely eaten by the foster mothers.

It was previously suggested [Hartwell, 1921, 1] that excess protein caused an alteration in composition of the milk, and finally a cessation of the flow. From these experiments it would appear that in some rats the secretion could be re-started, but not in others.

It is possible in some cases to alter the diet, either by adding milk, omitting the excess protein, or entirely changing the food to bread and milk, and thus to obtain recovery of the babies.

Experiments on changing litters.

That the milk of the rat on excess protein contains some toxic substance is further demonstrated by changing the litters.

Rat 136 received bread and milk. Rat 135 had a diet with excess protein. The litters were born on the same day. As soon as the babies 135 developed spasms (the 12th day) the litters were changed. Litter 135 recovered rapidly when fed by rat 136, which was having bread and milk, and were successfully weaned at the normal time. Two of litter 136 developed screaming fits and bad spasms the 20th day, but recovered. In another exchange the normal babies given to a mother, whose own litter were showing bad symptoms, lost weight for three days, after which they ate for themselves. In this experiment no typical symptoms were noticed.

Thus it is evident that with large amounts of protein in the diet, which is complete physiologically, the milk is affected, and the babies show abnormal and typical symptoms. It is suggested that

(i) The *quality* of the milk is altered, in that it possesses some toxic properties responsible for the spasms. This may be due to excess of amino-acids, or possibly to some deficiency in the milk.

(ii) The *quantity* of the milk is inadequate, since the babies lose weight.

Since large quantities of protein are usually recommended for a lactating animal, it is probable that definite and large amounts of one or more other constituents should be included in the diet.

With a view to throwing some light on this, the following experiments were made.

(ii) *Effect of adding whole milk to a diet containing excess protein.*

The bread and protein were fed in the same proportion as in the last series of experiments. The milk was heated to 70° C. In these experiments the rats had no water to drink. (In some earlier experiments, when 100 cc. whole milk were given, it was noticed that the animals drank no water, and usually upset the containing vessel as soon as it was given to them.)

The control experiment D was done at the same time of the year as the other experiments, though not actually the same year. So far the growth curves of the young when the mother is fed on bread and milk are practically constant, irrespective of the time of year, so long as the animals are kept in a well-heated room. Other observers find different growth curves according to the time of year. It is possible that this difference is minimised when the diet is exceptionally good, *e.g.* bread and whole milk.

In these experiments no abnormal symptoms of any kind were shown, but in all cases the growth was depressed, as compared with the standard (bread and milk).

This is well shown by the following figures:

Average weight of young rat at twenty-one days.

Exp.	Diet	Weight in g.
D	Bread and milk	39·2
41	" " edestin	31·3
108	" " edestin	29·6
J	" " caseinogen	27·8
C	" " caseinogen	30·6
201	" " gelatin	30·4
202	" " gelatin	26·0
203	" " egg albumin	29·0
204	" " egg albumin	34·3 (litter of three only)

In these experiments the rats were eating even larger quantities of protein, since milk contains from 2·5 %–4·0 % protein [Matthews, 1921]. The exact amount of milk required to prevent the babies from developing the typical symptoms has not been determined, but other experiments showed 50 cc. to be inadequate. In all probability the individuality of the rat would be a factor conditioning the amount required. Later in this paper (p. 101) it is demonstrated that the whey from 100 cc. milk is equally efficient in preventing bad results with caseinogen and edestin, and that the growth is equal to that obtained with bread and milk.

Thus milk contains some dietary constituents which render it possible for the rat to ingest even larger quantities of protein and yet the litter is not affected by bad symptoms. The only detrimental effect is that the babies do not grow at a maximal rate.

The next experiments were started with a view to finding the nature of such substance or substances.

Two experiments were made with each protein and the average weight of the two litters does not correspond very closely. In spite of the fact that no symptoms developed in the babies, the mothers were ingesting very large amounts of protein and it is probable (as before suggested) that the individual metabolism of the mother is a partial explanation of these discrepancies.

(iii) *Effect of adding other constituents to a diet containing excess protein.*

In this series the proportion of protein to bread was kept constant as in the previous experiments, *i.e.* 5 : 15.

(*a*) *Salts. Calcium* given as lactate, 1·0 g. per diem (Fig. 5).

Exps. 114, 172, 156. Bread, caseinogen, marmite, calcium lactate.
Exp. 138. Bread, egg albumin, marmite, calcium lactate.
" 140. Bread, edestin, marmite, calcium lactate.

Litter 114 grew well the whole time; they were excitable and exhibited toe-walking, but otherwise no typical symptoms. It is probable that the mother had a greater tolerance for protein, as before suggested. Apart from this litter, the results were in agreement and the same for each protein tried,

i.e. caseinogen, edestin, and egg albumin. All the litters showed typical and very bad symptoms, but the babies lived longer than usual.

Thus the addition of calcium lactate to a diet containing excess protein does not alleviate the symptoms but causes a slight prolongation of the life of the sucklings.

(*b*) *Salt mixture.* Bread, caseinogen, marmite, salt mixture. No improvement in the litter was obtained when 1·0 g. salt mixture was added to the daily ration. The symptoms were bad, and the young were all dead on the 15th day.

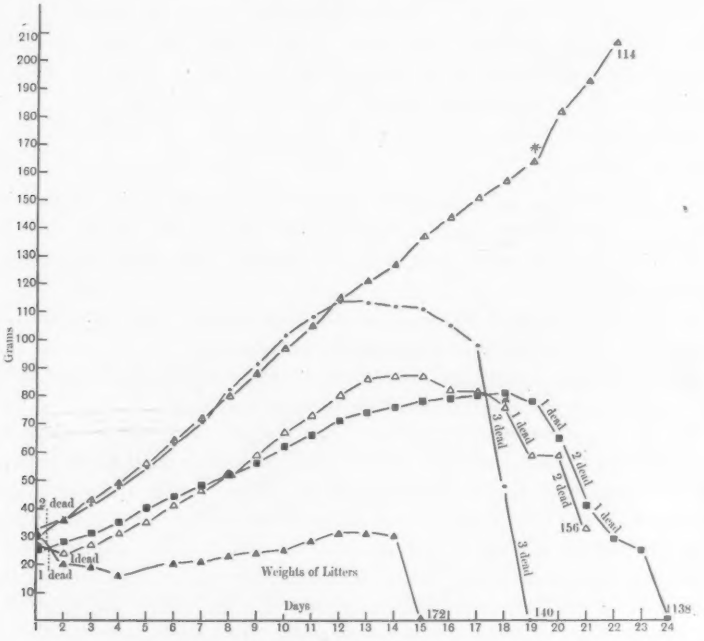

Fig. 5. Calcium Lactate.

(*c*) *Milk ash*[1]. The milk was evaporated to dryness, the residue scraped up and strongly heated in a crucible until a constant weight was obtained; 0·7 g. were given per day (*i.e.* the amount in 100 cc. milk).

These results were very similar to those obtained on adding calcium lactate to the excess protein diet. The symptoms were in no respect alleviated, but the life of the young was slightly prolonged.

(*d*) *Fat and vitamin A.* No beneficial effects were obtained on adding 3·5 g. butter per day to the excess protein diet. Edestin was used.

[1] I am indebted to Miss W. M. Clifford for the preparation of the milk ash.

(*e*) *Lactose.* 4·0 g. lactose were added to a diet containing excess edestin, but the spasms and bad effects were in no way improved.

(*f*) *Fat, vitamin A, and lactose.* Caseinogen was used and the diet was physiologically complete in all respects, fat and lactose being added in approximately the same amounts as found in 100 cc. milk.

The litter suffered very badly, two eventually survived, but they were weakly for some time and were not in a fit condition to leave the mother until the 26th day.

Vitamin C. The diet consisted of bread and egg albumin in the usual proportions and 0·7 cc. lemon juice per day. Great difficulty was experienced in getting a rat to bring up a litter on this diet. In many experiments the babies died within the first three days. The mothers were well, the babies were average weight, and apparently all right at birth. This may be pure coincidence. No explanation is offered to account for this failure.

Finally two experiments were successful. The litter all exhibited typical symptoms, and were dead by the 13th day.

(iv) *Effect of adding large quantities of yeast extract (commercial preparation, marmite) to a diet containing excess protein* (Fig. 6).

Two experiments were made with each protein (caseinogen, egg albumin and edestin).

1. The diet consisted of 15·0 g. bread, 5·0 g. protein, and 3·0 g. marmite, *all* constituents increased proportionately as necessary.

2. The bread, protein, and marmite were the same as above, but butter, lemon juice, and salt mixture were added to complete the diet.

These curves are very similar to those obtained when 100 cc. whole milk were added to the excess protein diet. To bring out this point curve D (bread and milk standard) is included in the figure. With edestin and caseinogen a depression of the growth curve was obtained, very similar to that obtained when the mother had these proteins + 100 cc. whole milk. The babies were practically normal, but slight toe-walking and excitability were noticed; these features, however, were very quickly recovered from. With egg albumin, on the other hand, the results were not so good. No spasms were noticed, but the babies were far from normal. They were all right at first, but on the 14th day they felt cold, were weak, inclined to roll over, thin and lethargic. They walked very badly and their movements were jerky and incoordinate. Two of the litter 180 died but the rest survived, as did all litter 182. The survivors were not normal for two weeks after weaning.

The average weights of the young rats are given below.

		Average weight at 21 days. g.
Exp. D	Bread and milk (standard)	39·2
„ 164.	„ edestin, marmite	34·7
„ 165.	„ „ „ butter, etc.	25·7
„ 179.	„ caseinogen, marmite	34·0
„ 181.	„ „ „ butter, etc.	30·7
„ 180.	„ egg albumin, marmite	22·0
„ 182.	„ „ „ butter, etc.	26·3

From these experiments it is clear that "something" in marmite is capable of obviating the bad results in the litters usually obtained with excess of caseinogen or edestin in the mother's diet. Also the actual weights of the babies are better when the mother's diet is lacking in fat, vitamin *A*, salt mixture and the antiscorbutic vitamin. This may be merely coincidence; a far greater number of experiments would be required before any conclusions could be drawn. It is interesting to note that similar results were obtained

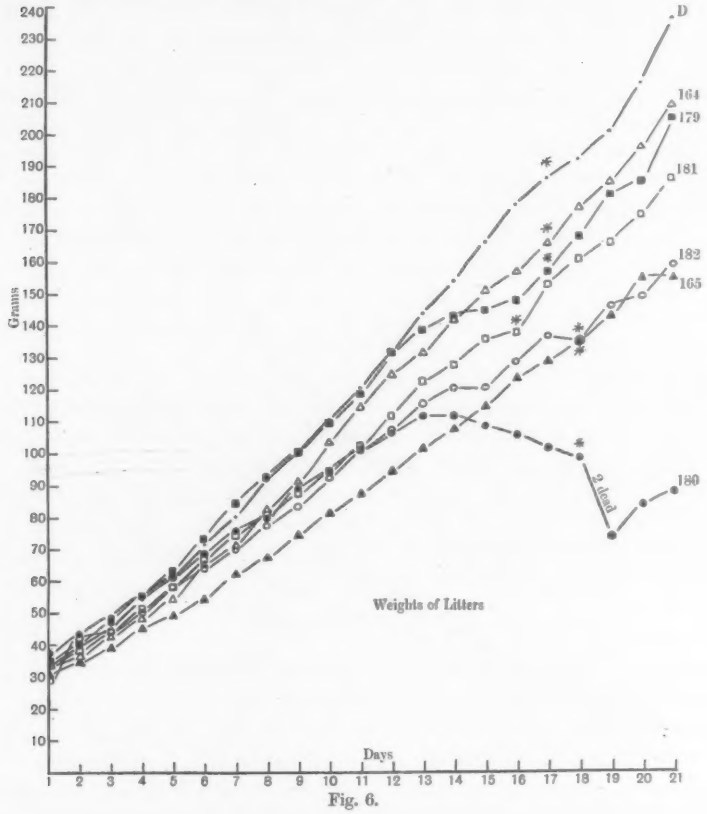

Fig. 6.

when less amounts of protein were given. Since the results are as good when the mother's diet is deficient in fat, vitamins *A* and *C* and salt mixture, it appears that the "something" in marmite is the primary factor. It may be of interest that the marmite contained only traces of calcium salts.

With egg albumin the improvement was not nearly so good. The litters certainly did better than when small amounts of marmite were fed to the

mother, but they could not compare at all favourably with the caseinogen and edestin babies. These differences are discussed later (p. 102).

The mothers maintained or increased their weights, except 180 (fed on egg albumin in a deficient diet) which lost appreciably.

(v) *The importance of protein.* (Fig. 7).

Exp. D.	Control. Bread and milk (same as in Fig. 6).		
Exps. 118, 120.	15·0 g. bread, 5·0 g. edestin		
Exp. 167.	,, ,, 3·0 caseinogen	}	+ whey from 100 cc. milk.
,, 168.	,, ,, 3·0 edestin		
Exps. 159, 162.	,, ,, 0·0 protein		
Exp. L.	Bread alone. [Taken from Hartwell, 1921, 1].		

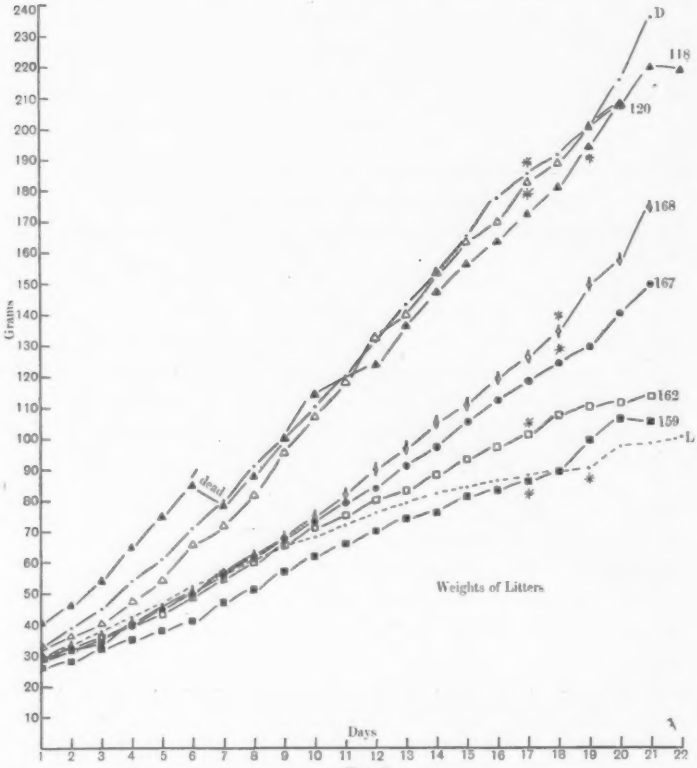

Fig. 7.

Preparation of the whey. 100 cc. milk were warmed to 40° C., and a few drops of rennet added. When the junket was firm, it was hung up in a muslin bag and the whey separated by dripping through. Each rat was given daily as much as could be obtained from 100 cc. milk. The protein and bread were increased proportionately as before.

It has already been mentioned that the whey was as effective as milk in preventing the bad symptoms in the litters. Also when the proportion of bread to protein was 15 : 5 the growth of the litter was practically equivalent to that obtained with a bread and milk diet, which up to the present time is regarded as maximal. It is extraordinary that such good growth should be obtained when the mother's diet was so poor in fat and vitamin *A*[1].

In this connection it is interesting to note that all the mothers that were killed had large deposits of subcutaneous and peritoneal fat. It may be that with large reserves of body fat, the amount in the diet is immaterial from the point of view of milk production.

In experiments 159 and 162 in which the mothers were fed on bread and whey, the litters did very little better than in experiment L when the mother received bread alone (taken from Hartwell [1921, 1]). Comparison of the protein rations in these experiments shows that the bread and whey contains only slightly more protein than bread alone. When more protein was added to the diet (experiments 167 and 168) a considerable improvement in growth of the young was obtained, though all the other constituents were the same as before. A further addition of protein (experiments 118 and 120) gave a still greater improvement in the growth of the litters. In this way it is seen that adding protein to the diet can raise the growth curve from almost the minimal to the maximal. Thus protein itself does play a very important part in the production of milk of good nutritive value, yet from other experiments described in this paper, it is obvious that the diet must be rich in other constituents as well or else harmful results occur in the litter.

One of litter 118 was killed accidentally on the 7th day and the average weight of these babies is rather greater than that of 120, whose mother received a similar diet. It is quite possible that the actual number in the litter is a factor of importance. Experiments to investigate this point are now in progress.

Discussion of Results.

(i) *Prevention of the bad symptoms in the young.*

It has been conclusively proved that large quantities of protein are by no means harmful to the rat itself, since the young and adult animals can live normally on a diet containing excess protein for considerable periods. The proportion of 15·0 g. bread to 5·0 g. protein proved an excellent diet for growing rats, but much less protein, even as little as 15·0 g. bread to 1·0 g. protein produced bad symptoms in the suckling litter. Therefore, it appears that the rat can metabolise the protein effectively from the point of view of its own growth, but is unable to produce normal milk, unless definite quantities of some other constituent (or constituents) are supplied. Our knowledge

[1] Since the caseinogen and edestin used were not absolutely pure, it is possible that traces of vitamin *A* may have been present.

of the metabolism of individual amino-acids is very limited, but the experiments described in this paper show that there is some quantitative relation between the amino-acids and other dietary constituents. What these constituents are is a very difficult problem. The fact that whole milk can prevent the bad symptoms throws very little light on the subject. Owing to the complicated nature of milk, it merely proves that large quantities of protein in the mother's diet produce no harmful effects in the litter, as long as "something" else is included. It is even possible that the necessary factor will vary with the nature of the protein.

It has been pointed out that 3·0 g. marmite to 5·0 g. protein is adequate in preventing spasms, etc., in the case of caseinogen and edestin, but with egg albumin, although the symptoms are somewhat reduced, the litter still suffers badly. Also with egg albumin much better results were found when small quantities of butter, lemon and salt mixture were added to the diet, but with caseinogen and edestin, it was immaterial if the diet was complete or not as long as large quantities of marmite were fed. Since caseinogen and edestin are somewhat similar in constitution, and egg albumin is different there seems some evidence for suggesting that the factor will vary with the constitution of the protein.

Some experiments with egg albumin now in progress show that the litter exhibit still greater improvements (but are *not* normal) when more butter is added to the ration, the diet being physiologically complete.

It is quite obvious that some other constituents are vitally essential for the production of normal milk, if large quantities of protein are ingested.

These are all present in milk, and in the case of caseinogen and edestin, present also in commercial yeast extract ("marmite"). In the light of present-day investigations the most obvious explanation is that vitamin *B* is the factor, but this conclusion is by no means justified from the experiments described here. Again "marmite" alone was not wholly effective with egg albumin. It might be that insufficient was given, but this is hardly likely, because 3·0 g. to 5·0 g. protein is a large proportion. A more probable explanation is that some other constituent is also needed for the proper metabolism of this protein in a lactating animal.

The fact that large quantities of milk and marmite were necessary suggests an actual chemical relation between the protein and some other factor, and not a catalytic or hormone action. It is possible that less marmite would be required if the right proportion of other constituents could be arrived at. Experiments have shown 1·0 g. marmite per day to be ineffective, yet the amount necessary to supply vitamin *B* to a rat is estimated as 0·2 g. per day.

It is possible that the animal needs a greater amount of vitamin *B* for the effective metabolism of large amounts of protein; hence it is used by the mother and she has none to spare for her milk. On the other hand the mother is usually regarded as being a safeguard for the young and it is thought that she can sacrifice her own tissues for the benefit of her offspring. Also it has

been proved that in a non-lactating animal quite small amounts of vitamin *B* are adequate even when the intake of protein is high.

The experiments of Karr suggest that vitamin *B* is an essential constituent of a dog's diet, otherwise the animal refuses its food. Since the dog is a carnivor, it might be assumed that this vitamin plays some part in connection with protein metabolism. Karr, however, states that the intermediary metabolism of nitrogen is unaltered by adding vitamin *B* to the diet, but he appears to deduce such a conclusion from analysis of excreta and food ingested. It is possible that the nitrogen excretion might remain constant and yet the intermediary metabolism be altered.

Thus there seems a certain amount of superficial evidence for associating vitamin *B* with protein metabolism. In the experiments described in this paper, all other recognised dietary constituents have been eliminated except vitamin *B*. This is only a negative proof and therefore of little value. The commercial preparation marmite is a somewhat crude product and contains many other substances besides vitamin *B*. It is even possible that extractives are of use in obviating the bad symptoms resulting from excessive amounts of protein in the mother's diet.

(ii) *The amount of protein.*

At present it is impossible to state what amount of protein is harmful and what amount is beneficial, because it appears to depend largely on the other dietary constituents. The experiments described in this paper show that too much protein depresses the growth curve, even if no bad symptoms are shown. For example when the mother is fed on bread and protein (15 : 5) and whole milk, the litter show a less good growth curve than when the mother is fed on bread and milk only, although the babies are normal in each case. In the former experiment the rat was taking at least 8·0 g. protein per day, but no bad symptoms occurred, yet in a less complete diet 1·0 g. protein can produce a very bad condition in the young.

As the results differ with different proteins it is probably certain amino-acids which are primarily responsible for the bad effects on the babies. If this is so, a diet of mixed proteins (such as taken by man) should prove less harmful, unless the protein is taken in very great excess. The actual composition of foods themselves is a safeguard, because it is rare to find only one protein.

(iii) *Development of the young.*

It has been pointed out that the time of onset of the symptoms is remarkably constant and it is therefore suggested that the stage of development of the young rat is a factor to be considered. In foster litters, the babies develop a little more slowly and their eyes open about two days later than those of normal litters. They do not eat for themselves until a few days later than the original babies and therefore cannot be weaned at the normal time.

(It has been found advisable to leave the mother with the young for at least three days after they can eat for themselves.) In general they seem more backward and less developed than normal babies of the same age. In foster litters, when the mother is fed on an excess protein diet, the symptoms usually develop about 2–3 days later, than in a litter left with its own mother. Also the symptoms come on at the same time, even if the mother has been fed with excess protein during gestation, or if she has first fed her own litter when she has had the excess protein diet. If the composition of the milk changed towards the middle and end of lactation, then the symptoms in the foster-litter should come on at an earlier stage, provided that the excess protein diet were continued throughout. But this is not so.

(iv) *The value of milk in the diet of a lactating animal.*

"Milk in *liberal amounts* should always be included in the diet of the lactating mother." This statement is made by McCollum [1920] and is probably a very true one. It was shown [Hartwell, 1921, 1] that bread and milk fed to the mother produced maximal growth of the young. Various other diets have been tried but as yet no curves have been obtained which are as good (except 15·0 g. bread to 5·0 g. protein + whey from 100 cc. milk—and this is a milk derivative). It has been proved in this paper that large quantities of milk entirely obviate the bad symptoms produced by excess protein in the diet. It is, therefore, possible that by taking large amounts of milk, the mother safeguards herself from any errors in diet.

The value of milk is, no doubt, due to the fact that it contains all the necessary constituents of a good diet, and not excessive amounts of any. It has been suggested by some physiologists that milk contains a specific galactogogue. Some experiments now in progress show that there are other diets which will give as good results as bread and milk (at any rate with rats) provided that the *right proportion* of the various constituents can be ascertained.

Another advantage of milk is that the proteins are adequate and it is quite obvious that the quality of the protein fed to a lactating animal is of primary importance.

SUMMARY.

1. In a lactating rat, the amount of dietary protein constituting excess varies with the type of protein and with the individuality of the rat. The proportion 15·0 g. bread to 1·0 g. protein (egg albumin or caseinogen) fed to the mother results in abnormal symptoms in the litters.

2. On a diet of bread and egg albumin (either 1·0 g. or 2·0 g. egg albumin to 15 g. bread) the mother loses a considerable amount, in one case as much as one-third of her body weight.

3. When excess protein is fed to the mother, the growth curves of the litters are not constant. The variations obtained are probably due to the individual metabolism of the different rats.

4. Large quantities of protein fed to a nursing rat are detrimental to the young, even when the diet contains all the essential constituents.

5. If such a diet is started at birth, some babies may survive, but they are not normal; the majority die.

6. If the diet is begun before or during gestation, none of the young survive.

7. The litters die at approximately the same time, irrespective of the length of time the mother has been fed on the excess protein diet.

8. Foster babies suffer in exactly the same way as the original family if the same diet is continued.

9. The typical symptoms can be induced in a healthy litter by giving them to a mother whose own litter has developed this condition.

10. It is suggested that the toxic substances in the milk affect the baby rat at some special stage of development.

11. A diet containing large amounts of protein (15·0 g. bread to 5·0 g. protein) in a physiologically complete mixture is adequate for growth, fertilisation and reproduction in the rat, and is unsuitable only during lactation.

12. The bad effects are entirely obviated by adding 100 cc. whole milk to the mother's diet, but the growth of the litter is not maximal. This applies to caseinogen, edestin, egg albumin and gelatin. With caseinogen and edestin, 100 cc. whey are equally effective.

13. With caseinogen, edestin and egg albumin the addition of calcium lactate to the diet prolongs the life of the young, but the symptoms are just as severe. Milk ash has a similar effect when added to a diet containing excess egg albumin or edestin.

14. The addition of butter and lactose causes no improvement in the young when the mother is eating large quantities of edestin or caseinogen.

15. Yeast extract (marmite) in large amounts added to the excess protein diet prevents all bad symptoms in the case of caseinogen and edestin; with egg albumin the bad condition is improved, but not entirely cured.

I wish to express my thanks to Prof. V. H. Mottram for his interest in this work, the expenses of which were defrayed by a grant from the Medical Research Council.

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XV. EFFECT OF SEVERE MUSCULAR WORK ON COMPOSITION OF THE URINE.

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A FAIRLY complete series of observations was undertaken to determine the effect of severe muscular work on the composition of the urine, using recent standard methods. These observations form part of a research connected with industrial fatigue, the subject being employed at the same time for observations on respiratory exchange.

In a previous paper [1921] we recorded the results of observations on day and night urine under various routines—five days' complete rest, five days' ordinary laboratory work, and five days' light muscular work, 67,500 kilogrammeters (kgm.) per day. In the present paper we give results obtained from the same subject, under similar conditions as regards diet, time of meals, etc., during five days' severe muscular work. The diet was controlled as regards quality. About ½ litre more fluid per diem was swallowed during the severe muscular work than during the other routines.

Our subject was accustomed to do 13,500 kgm. per hour, on a bicycle ergometer, working three hours before dinner—10 a.m. to 1 p.m.—and two hours in the afternoon—2 p.m. to 4 p.m.—the total, 67,500 kgm. producing only a slight degree of fatigue. For severe muscular work he attempted to do 100,000 kgm. per day at the rate of 20,000 kgm. per hour with the same interval for dinner. 100,000 kgm. represents a full day's work for a vigorous labourer, and, as might be expected, our subject—a laboratory assistant—found this amount of work a severe task. Table I shows the details of the work actually performed. On the first day the subject had to give up the attempt at the end of the third hour, the first hour's work of 20,000 kgm. affecting him very much. On the second day he was able to do more and on the third, fourth and fifth days he completed the full amount in the five hours. It was obvious from the subjective and objective symptoms that the experiment produced severe strain. The average composition of the 24 hours' urine for the five days is shown in Table II; the significance of any of the five days' results was similar. For comparison we have given the figures for the 24 hours' urine obtained in our previous research [1921]. It will be observed that the undetermined nitrogen, creatinine, neutral

sulphur—and, therefore, the total sulphur—and lactic acid were distinctly increased during the severe muscular work. Acetone bodies were present during the third and fourth days and the third night, probably indicating complete consumption of available carbohydrate. Purine nitrogen was also increased, but we had not complete figures for comparison. Other observers have obtained somewhat similar results. Cathcart [1921] reviews the most important of the recent and older researches in his monograph on protein metabolism.

Table I.

Date	Work done in kgm.	Working period in hours	Remarks
10. x. 21	44,482	3	Felt very hot, perspired freely; very much fatigued; severe headache; sore all over
11. x. 21	72,440	5	Felt very hot, perspired freely; very much fatigued; felt stiff all over
12. x. 21	104,760	5	Felt very hot, perspired freely; very much fatigued; felt stiff all over; acetone bodies in urine
13. x. 21	100,000	5	Not so hot; not so fatigued; not so stiff as on previous days; acetone bodies in urine
14. x. 21	100,000	5	Not so hot; not so fatigued; not so stiff as on previous days

Osterberg and Wolf [1907] showed that the undetermined nitrogen was increased during activity. Shaffer [1908] found that muscular activity—walking ten miles—did not increase the excretion of creatinine. Pekelharing and Harkink [1911] obtained a similar result, but observed that after prolonged tonic contraction the output rose. Our subject exhibited muscular stiffness and this may have been a cause for the increased excretion of creatinine. Hoogenhuyze and Verploegh [1908] found that in fevers and other pathological conditions in which there is an increased breaking down of tissue, the creatinine excretion is increased. In our subject there was the possibility that some muscle fibres were injured. The brain contains a fair proportion of creatine [Janney and Blatherwick, 1915], and was a possible seat of production of creatinine during the severe fatigue produced. Weinberg [1921] considers that an important influence on the excretion of creatinine in the urine should be assigned to the mind, high excretion being connected with emotion. Scott and Hastings [1920], Garrat [1898] and others noted that there is a rise in output of inorganic sulphate during activity. Although we observed a similar increase of sulphate during the light muscular work it was the neutral sulphur that was most increased during the severe work. This may have been due to the want of oxygen, so that the sulphur was not so completely oxidised during the severe work. With regard to lactic acid, many observers have found that this acid is increased by severe muscular exercise. Fletcher and Hopkins [1907] clearly demonstrated that lactic acid is produced in excised muscle only when the muscular contraction occurs in a deficiency of O_2. When it occurs in an adequate supply of O_2, CO_2 instead of lactic acid is produced. Hill and Flack [1909] showed that in the fatigue of athletes oxygen inhalation increases the lasting power and decreases fatigue, probably

by maintaining or restoring the vigour of the heart. This leads to the question whether creatinine, neutral sulphur and undetermined nitrogen were increased because of insufficient oxidation. This was a possibility, but in one respect these substances differed greatly from lactic acid; lactic acid was excreted in much greater amount during the day (Table II) than at night, whereas creatinine, neutral sulphur and undetermined nitrogen were more evenly distributed between day and night. With regard to the latter substances we did not notice in our previous researches [1921] that there was any parallelism in these excretions; but, nevertheless, there may be the same explanation for

Table II.

	Average 24 hours results				Average hourly day and night results during severe muscular work	
	Complete rest	Laboratory work	Light muscular work (67,500 kgm. in five hours)	Severe muscular work (100,000 kgm. in five hours)	Day	Night
Amount cc.	1112	1116	971	686	32·4	25·9
Acidity %	49·5	53·7	58·0	65·0	60·0	70·0
Titratable acidity (Folin) cc. *N*/10	309	312	332	342	14·0	14·4
Total acidity cc. *N*/10	703	673	723	722	29·0	30·7
Total N g.	8·010 (100)*	9·015 (100)	9·465 (100)	10·250 (100)	·446 (100)*	·414 (100
Urea N g.	5·990 (74·80)	7·131 (79·10)	7·647 (80·80)	7·680 (74·93)	·336 (75·3)	·308 (74·
Ammonia N (A) g.	·439 (5·48)	·385 (4·27)	·442 (4·67)	·412 (4·02)	·015 (3·4)	·019 (4·6
Ammonia N (B) g.	·548	·505	·552	·533	·021	·023
Amino-acid N g.	·109 (1·36)	·120 (1·33)	·110 (1·16)	·121 (1·18)	·006 (1·4)	·004 (1·0
Creatinine N g.	·741 (9·25)	·736 (8·17)	·695 (7·34)	·925 (9·02)	·036 (8·2)	·040 (9·6
Uric acid N g.	·154 (1·92)	·141 (1·56)	·133 (1·40)	·134 (1·31)	·006 (1·4)	·005 (1·2
Undetermined N g.	·578 (7·19)	·502 (5·57)	·438 (4·63)	·978 (9·54)	·047 (10·3)	·038 (9·1
Chloride (NaCl) g.	9·410	7·207	8·801	6·736	·394	·200
Phosphate (P_2O_5) g.	1·550	1·703	1·711	1·811	·070	·080
Total S (SO_3) g.	1·594 (100)	1·749 (100)	1·770 (100)	2·069 (100)	·084 (100)	·087 (100
Inorganic S (SO_3) g.	1·148 (72·0)	1·400 (80·0)	1·432 (80·9)	1·422 (68·7)	·057 (67·9)	·061 (70·
Ethereal S (SO_3) g.	·212 (13·3)	·132 (7·5)	·140 (7·9)	·209 (10·1)	·007 (8·3)	·009 (10·
Neutral S (SO_3) g.	·234 (14·7)	·227 (12·5)	·198 (11·2)	·438 (21·2)	·020 (23·8)	·017 (19·
Lactic acid g.	trace	trace	trace	·061	·0040	·0014
Calcium (CaO) g.	—	·281	·252	—	—	—
Magnesium (MgO) g.	—	·173	·156	—	—	—
Purine N g.	·035	—	—	·058	·0023	·0025

(A) Van Slyke's method. (B) Malfatti's method.

* Figures in brackets are percentages. Total acidity is Ammonia (B) + titratable acidity.

the increase of each during severe work. It is well known that creatinine and neutral sulphur resemble one another in that they are of endogenous origin, and that they are practically constant in amount for each individual, being evidently connected with the mass of active living protoplasm. In our subject both creatinine and neutral sulphur were higher on the first day than on the succeeding days of severe work. We consider that the probable explanation of the increased excretion of creatinine, neutral sulphur and undetermined nitrogen was damage to protoplasm both nervous and muscular by the excessive fatigue and strain.

Cathcart [1921] states that no one has been able to demonstrate clearly that muscular work affects greatly the total nitrogen excretion, provided the

supply of food, particularly of carbohydrate and of oxygen be sufficient. Under our conditions of experiment we found a slight and steady increase in total nitrogen from complete rest to severe work (Table II). We also found that during the five days' severe work the total nitrogen rose from 8 g. on the first day to 10 g. on the second day, to 12 g. on the third day and fell to 10 g. for the fourth and fifth days, whereas the increments in undetermined nitrogen, neutral sulphur, creatinine were marked from and including the first day. Garratt [1898] found that a rise of sulphate preceded a rise in total nitrogen as a result of exercise. Whilst the figures for total nitrogen are of interest and of some significance and show that the excretion of total nitrogen was increased, we cannot conclude that there was an increased katabolism of nitrogen in our subject as the result of exercise, since we did not estimate the total nitrogen in the food and in the faeces. Under ordinary conditions our subject's total nitrogen averaged only 9 g. per diem during six months and was never as high as 12 g. The large amount of nitrogen, 12 g. on the third day of severe work, was probably due to lack of carbohydrate as indicated by the presence of acetone bodies on that day. Many observers have found an increase of nitrogen when there is an inadequate supply of nitrogen-free food available.

Because it was necessary to increase greatly the amount of work for our subject before any definite change in composition of urine was detected we consider that under equally good dietary and atmospheric conditions a healthy industrial worker would not show any such change in urinary composition unless undertaking work far more severe than his customary daily task. We have done a few experiments with some subjects sitting at rest under hot (36° C.) and close atmospheric conditions and found that the day urine showed higher figures for titratable acidity and ammonia than when under comfortable atmospheric conditions. We hope to extend these observations. One of us [J. A. C.] has noted high excretion of ammonia in a hot climate [1919, 1920].

Table II also gives the average hourly day and night results during the five days' severe work. As in our previous research [1921] we found that the acidity, ammonia and phosphate were higher at night than during the day; also that the sulphur was evenly distributed between day and night, whilst the total nitrogen was higher during the day than at night. We consider that during the night there is an excretion of fixed acids which have been formed in the cells during the day and that these fixed acids are concerned in the production of fatigue and sleep. For all routines examined we observed that the differences between day and night urine were most marked during rest in bed and less marked as the activity increased, activity probably hastening the processes concerned.

We are indebted to Mr C. Pergande, who was the subject in this research.

SUMMARY.

A healthy subject, who was accustomed to do 67,500 kgm. of work, on a bicycle ergometer, in five hours, developed symptoms of muscular strain on attempting to do 100,000 kgm. of work in five hours and showed pathological changes in urinary composition. Creatinine, undetermined nitrogen, neutral sulphur and lactic acid were much increased, whilst acetone bodies were present during part of the experiment.

Our previous results regarding composition of day and night urine were confirmed.

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XVI. THE VALUE OF GELATIN IN RELATION TO THE NITROGEN REQUIREMENTS OF MAN.

By ROBERT ROBISON.

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The story of the earliest attempts to discover the value of gelatin as a foodstuff has been told by Carl Voit [1872] in the introduction to his paper on this subject. It commences in 1682 when Dionys Papin prepared gelatin from bones by means of his digestor, and from the gelatin made soup with which he fed the poor. Such attempts were zealously renewed during the French Revolution by Cadet de Vaux, d'Arcet and others, and were supported by the Government, who issued official instructions extolling the nourishing properties of gelatin soup above those of beef tea. The approbation of the Institute of France and of the Academy of Medicine was also forthcoming, but in spite of d'Arcet's attempts to improve the flavour of the soup with spices, it did not meet with very great approval from the poor, who were expected to consume it.

Gannal, a manufacturer of gelatin, fed himself and his family on gelatin, with and without bread, for some weeks until compelled to desist owing to the unsupportable nausea caused by the diet. The effects on the health of these people led him to conclude that gelatin is not only valueless as a food but actually harmful.

Magendie's Report in 1841 to the Paris Academy on the results of the investigations of the second Gelatin Commission was scarcely more favourable. Gelatin was considered to have no food value by itself and to reduce the value of other foodstuffs when fed in combination with them. A similar opinion was expressed by the Academy of Medicine in 1850 but less extreme views were held by some physiologists among whom were Boussingault [1846] and Frerichs [1845]. The latter ascribed to gelatin the same significance as that of the "Luxus" protein, *i.e.* the excess protein in the diet over the requirements of the body as represented by the protein decomposition during starvation. Though unable to replace the body protein gelatin could, he held, be utilised in the same way as the nitrogen-free foodstuffs ("Respirationsmitteln").

Somewhat similar views were held by Bischoff [1853] while Donders [1853] considered that gelatin might reduce the body's needs for protein since these are not restricted merely to the replacement of tissues.

Voit's own experiments and those carried out in conjunction with Bischoff on dogs form the first systematic study of the nitrogen balances on diets containing varying quantities of meat, gelatin and fat. As the result of a large number of experiments he concluded that gelatin always spares protein and in a greater degree than fat or carbohydrate, but that gelatin plus fat reduces the protein decomposition more than does gelatin alone. On the other hand, however much gelatin and fat are given, body protein will be lost.

The energy requirements of the animal were not sufficiently considered in these investigations and a large part of the so-called sparing action of gelatin can be ascribed to its ability to furnish energy and so to reduce the use of body protein for this purpose. I do not suggest that the whole effect is to be explained in this way, but to what extent gelatin can satisfy any portion of the dog's specific nitrogen requirements cannot be ascertained from Voit's results.

During the next thirty years experiments upon dogs by feeding with gelatin were also carried out by Oerum [1879], Pollitzer [1885], Munk [1894], Kirchmann [1900] and Krummacher [1901]. In Oerum's experiments the dog received a basal diet of starch, butter and meat extract equivalent to over 80 calories per kilo body weight. During successive periods of from four to eight days this diet was supplemented by meat or the equivalent amount of gelatin. Unfortunately only the urea nitrogen was determined (by Liebig's titration method) and the faeces were not analysed, so that a nitrogen balance sheet cannot be made out, but the results appear to indicate that gelatin can save about half the amount of nitrogen excreted by a dog when receiving a carbohydrate diet of sufficient calorie value.

Pollitzer also gave his dog abundant carbohydrate to which, during successive periods, were added equivalent quantities of meat, digestion products of meat (peptone, etc.) and gelatin. Positive nitrogen balances were obtained with all except gelatin.

Kirchmann determined the amount of body protein spared by different amounts of gelatin, no other food except water being given. Taking the nitrogen output during starvation as 100 he found a saving of 25 % when the gelatin given was sufficient to satisfy only 7·5 % of the energy requirements of the animal, while eight times this amount was required to save 35 %. He estimated that a maximum saving of 39 % might be expected if the amount of gelatin could be increased to meet these energy requirements in full.

Krummacher continued these experiments with still greater quantities of gelatin, and obtained a result closely agreeing with Kirchmann's calculated figures. It is clear however that unless we also know the minimum nitrogen output when the energy requirements are fully met by nitrogen-free foodstuffs, the above relationships offer no evidence as to the capacity of gelatin to satisfy any of the specific nitrogen requirements of the animal. This value was not determined by either Kirchmann or Krummacher.

Munk's experiments were on a different plan from those mentioned above.

He gave a dog a diet of rice, fat and meat equal to 58 calories per kilo and containing 0·6 g. nitrogen per kilo body weight, which was more than twice the starvation output. He was then able to replace five-sixths of this protein by the equivalent amount of gelatin and still keep the animal in nitrogen equilibrium.

Kauffmann [1905] carried out a series of experiments on a similar plan but with the precaution of reducing the nitrogen intake of the standard diet to a much smaller amount than that given by Munk. This standard diet consisted of milk, rice, caseinogen (plasmon) and fat and was given in amount equal to 0·32–0·39 g. N and 63–72 calories per kilo body weight. Not more than one-fifth of the nitrogen of this diet could be replaced by gelatin nitrogen without an increase in the nitrogen output occurring. Kauffmann also investigated the possibility of improving the value of gelatin by supplementing it with tyrosine, tryptophan and cystine and concluded from his experiments on dogs and on himself that with these additions gelatin becomes of equal value with caseinogen.

At a much earlier date Escher [1876] had fed dogs and pigs with gelatin supplemented by tyrosine and found that their body weight was maintained, but Lehmann [1885] was unable to obtain this result in experiments on rats.

Rona and Müller [1906] carried out a series of very careful experiments with dogs on the same plan as those of Kauffmann but were unable to confirm the latter's conclusions. Their standard diet gave 0·2 g. N and 91 calories per kilo body weight. With this the animal was in nitrogen equilibrium, but when a portion of the milk was replaced by gelatin plus tyrosine and tryptophan a negative balance was found.

The value of gelatin fed in conjunction with other proteins has also been investigated by Murlin [1907, 1] both by experiments on dogs and on himself. With dogs on a diet containing one-fourth more than the fasting requirement of nitrogen, half of this being in the form of cracker meal and half in the form of caseinogen, it was not possible to replace the caseinogen nitrogen by gelatin nitrogen without increased loss of body protein. With other diets however, in which the protein was in the form of meat, up to 58 % could be replaced without loss of body protein. The fuel value of all diets was greater than the energy requirements of the animal but Murlin attributes the high replacement value obtained in some diets largely to the greater proportion of calories supplied by carbohydrate in place of fat.

It is possible that this factor may have influenced the result, though according to Zeller [1914] the nitrogen requirements are not affected by the proportion of fat to carbohydrate in the diet so long as this does not become greater than about 4 : 1.

There seems however to be insufficient reason for assuming that the amount of meat given in some of these diets was the minimum required for nitrogen equilibrium, and unless this were so the fact that a part could be replaced by gelatin without affecting the balance would prove nothing.

On the other hand, on the cracker meal diets a negative balance was always obtained, which was recognised by Murlin as evidence of the lower availability of this form of protein. On these diets it was not possible to replace any part of the protein by gelatin without increasing the relative loss of body nitrogen.

The criticism that the protein in the diet after part of it had been replaced by gelatin may have still been in excess of the minimum required, applies even more forcibly to the experiment on himself, in which the basal diet contained 14·25 g. N, *i.e.* about 10 % more than his nitrogen output during starvation. When two-thirds of this had been replaced by gelatin nitrogen he was still receiving 5·33 g. N (0·076 g. per kilo) derived from eggs, cream, butter and cereals. During the two days on which this diet was taken a positive balance was obtained, but this cannot be accepted as convincing evidence of the value of gelatin nitrogen.

In a later paper Murlin [1907, 2] brought forward satisfactory proof that in a dog the reduction (about 30 %) of the fasting nitrogen output produced by small amounts of gelatin, was much greater than could possibly be accounted for by the dextrose which might be synthesised in the body from this gelatin[1].

From the investigations so far considered it may be taken as definitely established that:

1. Gelatin when given as the sole source of nitrogen is unable to maintain the animal body in nitrogen equilibrium.

2. With dogs gelatin is able to reduce the loss of body nitrogen considerably below that occurring during starvation, and this effect is not proportional to the amount of potential energy thus supplied and cannot therefore be simply explained on these grounds.

3. Some of the experiments indicate that when gelatin is mixed with other proteins, they may complement one another so that a proportion of the nitrogen of gelatin is utilisable.

A critical examination of the results of these experiments does not enable us to form any definite conclusions as to the capacity of gelatin alone to satisfy any part of the specific nitrogen needs of the body in man, although some of the results obtained with dogs indicate a limited capacity in this direction if the nitrogen output on an abundant nitrogen-free diet is taken as representing these specific requirements. There is however a difficulty in accepting this since the results obtained with dogs do not fall into line with those obtained with man and some other animals, and suggest that the nitrogen metabolism of the carnivora varies from that of the omnivora and herbivora in some details.

The fasting output of a man is equal to about 0·2 g. N per kilo body

[1] A brief account of other researches by Ganz, Gerlach (1891), who investigated the value of gelatin peptones, Gregor (1901), who used gelatin for feeding infants, and by Brat (1902) and Mancini (1905), who fed it to convalescents, will be found in Murlin's paper [1907, 1].

weight, that of a large dog is of the same order. On an abundant carbohydrate diet the nitrogen output of man can be reduced to one-quarter of this amount, *i.e.* 0·05 g. N per kilo whereas according to most observations under the same circumstances the nitrogen output of a dog is only reduced by 10 % to 20 %.

The difference in detail between the nitrogen metabolism of man and dog also emerges on comparison of the ratios of the total nitrogen to that excreted in the form of creatinine during starvation and on abundant nitrogen-free diets (see Table I). The constancy of the creatinine output and its probable relationship to the endogenous metabolism has been noted by Folin [1905], McCollum [1911], Zeller [1914] and others.

Table I

Observer	Animal	Weight Kg.	Diet	Total nitrogen in urine per kilo body weight	Creatinine nitrogen per kilo body weight	Total urine N / Creatinine N
Cathcart [1907]	Man V.B.	62·0	Fasting, 4th day	0·221	0·0056	39
,, ,,	,, ,,	60·0	,, 8th ,,	0·159	0·0053	30
Benedict and Osterberg [1914]	Dog 39	7·6	,, 3rd ,,	0·360	0·0099	36
,, ,,	,, 33	12·7	,, 3rd ,,	0·280	0·0112	25
Towles and Voegtlin [1912]	,, 3	9·0	,, 2nd ,,	0·294	0·0129	23
Murlin [1907, 2]	,, *C*	13·0	,, 4th ,,	0·257	0·0080	32
Folin [1905]	Man H.B.H.	85·7	Starch, cream, 1 g. N	0·0420	0·0070	6·0
Graham and Poulton [1912]	,, G.G.	62·4	Starch, cream, ·912 g. N	0·0445	0·0093	4·8
,, ,,	,, E.P.P.	72·4	Starch, cream, 1·23 g. N	0·0468	0·0107	4·4
af Klercker [1907]	,, a.K.	88·0	Low N	0·0319	0·0079	4·0
Robison [1922]	,, C.J.M.	60·5	Carbohydrate, fat, ·3 g. N	0·0352	0·0072	4·9
,, ,,	,, R.R.	58·0	,,	0·0355	0·0084	4·2
McCollum [1911]	Pig	10·9	Carbohydrate	0·0495	0·0095	5·2
,, ,,	,,	68·4	,,	0·0387	0·0069	5·6
Mendel and Rose [1911]	Rabbit	1·74	,,	0·126	0·0172	7·3
Murlin [1907, 2]	Dog *C*	11·3	,,	0·158	0·0104	15

There is a close parallelism between the figures for men and dogs during starvation and between those for men, pigs and rabbits on abundant nitrogen-free diets. The creatinine excretion for Murlin's dog *C* on such a diet is also in good agreement with the corresponding figures for men and pigs but the ratio $\frac{\text{Total urine N}}{\text{Creatinine N}}$ is about three times as high as the same ratio for other animals. It is of course not possible to state on such evidence alone that the real endogenous metabolism of this dog should be represented by a nitrogen output of one-third the observed amount, but it is clear that the nitrogen metabolism of dogs differs in some way from that of man, and that caution must be used in applying conclusions from experiments with these carnivora to other animals and man.

These criticisms however do not apply to the experiments of McCollum [1911] on pigs, for in these the constancy of the proportion of the endogenous

metabolism represented by creatinine nitrogen was recognised and was used as a criterion for judging when the minimum nitrogen excretion of the animals had been reached.

The pigs were fed on a basal nitrogen-free diet of ample fuel value consisting of starch, a salt mixture and water, until the nitrogen output had reached the minimum, whereupon an amount of the protein under examination equivalent to this minimum (urine nitrogen only) was added to the diet during a further period, after which the basal diet alone was fed until the output had again fallen to the minimum, the nitrogen excreted during this last period being also included in the calculation. In the experiment recorded by McCollum 2·62 g. of gelatin nitrogen was given daily during eight days, *i.e.* 20·96 g. in all. The total output during these eight and the following four days on which no nitrogen was given, amounted to 41·71 g. in the urine and 12·48 g. in the faeces, *i.e.* 54·19 g. in all, making a negative balance of 33·23 g.

Had the pig received no nitrogen at all its total output during these twelve days would have amounted to 31·44 g. in the urine and 12·48 g. in the faeces, making 43·92 g. in all, so that a saving of 10·69 g. nitrogen has been effected by 20·96 g. of gelatin nitrogen. This implies a utilisation of 50 % of the nitrogen given in this form, which was confirmed by five other similar experiments the details of which are not given. If the result is stated in terms of body protein saved, this amounts to 1·34 g. per day (if reckoned on eight days), *i.e.* 37 % of the minimum output in urine and faeces or 51 % of that in the urine only, which is taken by McCollum as representing the essential tissue metabolism of the animal.

Boruttau [1919] has recently attempted to determine the biological value of gelatin by two experiments on dogs, using the method and formulae adopted by Karl Thomas [1909] and has obtained the figures 49·1 % calculated by formula I and 67·3 % calculated by formula II. These values would agree much more closely had Boruttau not made an error in his use of formula I by taking the total food nitrogen as denominator in place of this amount less the nitrogen of the faeces, as intended by Thomas. In any case however such figures have no real significance in the case of gelatin since they will necessarily vary with the amount of the intake, and moreover the experiments were of too short duration to possess much value.

Apart then from the experiments of McCollum no very satisfactory evidence has been produced regarding the value of gelatin alone to satisfy any of the nitrogen requirements of the animal body. Most of the investigations have in fact been concerned with its value when fed in conjunction with other proteins and this introduces the possibility of complementary effect, about which very little is definitely known. That such effect is possible is shown by the experiments of Osborne and Mendel [1912] on rats. With gelatin as the sole protein the animals rapidly declined in weight but recovered when half of the gelatin was replaced by gliadin, a protein incapable of inducing more

than a very slight growth when fed as the sole protein constituent of the diet. Further, almost all the previous work, including that of McCollum, has been carried out on animals, and the results might not necessarily apply to man. The whole question is of very great theoretical and practical interest because of its bearing on protein metabolism in general and the nature of the body's requirements for particular compounds of nitrogen.

Experimental.

The investigation about to be described was an attempt to obtain more light on the problem by direct experiments on man.

The subject of the experiment was myself, age 37 years, medium build, weight 59 kilo, height 173·5 cm. My minimum nitrogen output had been determined by previous experiments which will be discussed in another paper.

In the second of these experiments, in which a diet containing about 0·3 g. N and equivalent to 2600–3000 calories (45–52 cals. per kilo) was taken for a period of seven days, the nitrogen output in the urine fell to a fairly constant level of 2·06 g., while the average amount of nitrogen excreted in the faeces was 1·13 g. per day.

In the present investigation the basal diet supplemented by different quantities of gelatin was taken for periods of ten days, the nitrogen intake being kept absolutely constant during each period.

Profiting by the experience of the previous experiments the basal diet was somewhat altered, the original attempt to introduce some variety and palatability being given up in favour of greater simplicity and uniformity of the food intake. The proportion of calories supplied by fat and the total nitrogen in the diet were both reduced. In the later experiments the process of simplification was carried to its furthest extent, the diet consisting of corn starch, lactose, sucrose and a salt mixture. Minimal quantities of lemon juice and cod liver oil were added to supply the antiscorbutic and fat soluble *A* accessory factors and agar-agar was taken to increase the bulk of the faeces and prevent constipation. The corn starch, lactose, salt mixture and agar for each day's ration were weighed out and mixed together before the experiment began. The mixture was taken in the form of a cream made with cold or warm (but not boiling) water and washed down with more water. The uncooked starch grains were very well absorbed, extremely few being found in the faeces. Usually a third of the day's ration was taken at 8 a.m., 1 p.m. and 7 p.m., but sometimes it was found necessary to increase the number of meals in order to consume the prescribed amount. The gelatin was dissolved in warm water and taken either by itself or mixed with some of the starch and lactose. The lemon juice, sweetened with cane sugar, was taken as a drink and a little weak tea with lemon was also permitted. The very small amount of nitrogen in the tea was assumed to be due to caffeine and to be excreted unchanged in the urine. It was therefore always subtracted from the total nitrogen intake and from the output.

The salt mixture had the following composition:

Calcium diacid phosphate $CaH_4(PO_4)_2$, H_2O	20	Na	2·5 %
Calcium lactate $(C_3H_5O_3)_2Ca$, $5H_2O$	30	K	13·5
Potassium hydrogen phosphate K_2HPO_4	30	Ca	7·1
Sodium dihydrogen phosphate NaH_2PO_4, H_2O	15	P	13·6
Magnesium carbonate $MgCO_3$	3	Mg	·85
"Iron carbonate"	2	Fe	·7

It was intended that 10 g. of this mixture with the addition of 5 g. sodium chloride should be taken daily. The amounts of calcium and phosphorus would then correspond with those recommended by Sherman as 50 % above the minimum requirements of the body for these elements [Sherman, Wheeler, and Yates, 1918; Sherman, 1920]. It was found necessary however to reduce these quantities to 6 g. and 4 g. respectively on account of the diarrhoea caused by the diet. The ash of the above salt mixture is markedly alkaline, a point of importance in view of the observations by McCollum and Hoagland [1913] on the increased nitrogen output caused by diets having an acid ash.

The urine was collected from 8 a.m. to 8 a.m. and stored under toluene. The faeces were collected over the whole period and mixed with dilute sulphuric acid, those passed during the morning being considered as belonging to the previous day. Owing to the fluid consistency of the faeces the use of markers was found to be impracticable, but in view of the regular evacuation of the intestines and the length of the experiment, no serious errors can have been introduced in this way. Estimations of nitrogen in urine, faeces and in all components of the diet were carried out by the Kjeldahl method in duplicate. Creatinine was estimated by the method of Folin.

The percentages of nitrogen found in the constituents of the diet and their fuel values are given in Table II.

Table II.

	Nitrogen per 100 g.	Calories per 100 g.
Gelatin (Coignet's "Extra." Gold Label)	14·16	324
Corn starch (*a*)	0·039	360
" " (*b*)	0·027	360
Dextrin	0·065	360
Agar-agar	0·242	—
Lactose	0·013	370
Sucrose	—	395
Butter	0·080	775
Cod liver oil	Not determined	930
Lemon juice (per 100 cc.)	0·067	40
Vermouth "	0·005	140
Tea infusion* "	0·008	—

* The strength of the tea infusion was kept as nearly constant as possible but the total nitrogen intake from this source was checked by removing an aliquot portion of all tea drunk during an experiment and estimating the nitrogen in the whole quantity.

Concerning the purity of the gelatin used in the experiments.

The source from which commercial gelatin is obtained and the methods employed in its manufacture are not likely to produce a pure product. One would expect to find it contaminated with traces of other animal proteins or their decomposition products. Such included impurities being colloids could not be removed by washing, and might conceivably possess a high value for the replacement of body nitrogen.

Kirchmann drew attention to the fact that the best French gelatin gave a slight precipitate with Millon's reagent and with potassium ferrocyanide and acetic acid, and claimed to have succeeded in removing the impurities to which these reactions were due. The unoxidised sulphur was also reduced from 0·387 % to 0·263 %. He considered that the difference between his own results and those of previous workers was to be attributed largely to the presence of this protein in their gelatin. One of his methods consisted in soaking the gelatin first in water, then in 10 % sodium chloride solution, again in water and finally in alcohol. Murlin [1907, 1], using the same methods, was unable to detect any improvement in the purity of the product.

The gelatin used in the present investigation also gave a slight positive reaction with Millon's reagent and with potassium ferrocyanide and acetic acid, and an attempt was therefore made to purify it by soaking it for 24 hours in *N*/20 HCl followed by *N*/20 NaOH, then for some days in running water. No appreciable reduction in the intensity of the colour produced with Millon's reagent was observed after such treatment.

Folin and Denis [1912] have recorded finding a trace of tyrosine in gelatin, using Folin's colorimetric method, and Dakin [1920] has recently obtained a similar result using a gravimetric method. He estimates the amount of tyrosine at about 0·01 % and considers that it cannot be an integral part of the gelatin molecule.

I attempted to estimate the amount of tyrosine present by means of Folin's method, using relatively large quantities of gelatin. The tyrosine in a sample of dried ox muscle was also estimated by the same method. The results are shown in Table III. Millon's reaction is not well adapted for colorimetric measurement but under suitable conditions it was found possible to make approximate determinations by comparing the colour with that developed by different amounts of pure tyrosine, and the results agree reasonably well with those obtained by Folin's method.

Table III.

	Tyrosine estimated by Folin's method	Tyrosine estimated with Millon's reagent
Gelatin (Coignet's extra)	0·57 %	0·6 % to 0·7 %
„ after purification	0·45	—
„ Swiss	0·55	—
Glue	1·47	—
Ox muscle	5·8	—

The accuracy of Folin's method has been called in question by Abderhalden [1913, 1, 2] who has suggested that other amino-acids, tryptophan, hydroxytryptophan and hydroxyproline give the same colour reaction. Of these the first two are not present in gelatin but Dakin estimated the amount of hydroxyproline as 14·1 %. Through the kindness of Prof. Leathes, F.R.S., who supplied me with a specimen of this amino-acid, I was able to test its behaviour with Folin's reagent and found that a slight colour developed under the conditions laid down by Folin and Denis for the estimation of tyrosine, but that the intensity was about $\frac{1}{375}$ of that produced by the latter compound.

A slight colour, similar to that given by hydroxyproline, was also obtained from a specimen of phenylalanine.

In the face of the results given by gravimetric methods it would be rash to assert that the gelatin actually contained 0·57 % of tyrosine, but the colour produced with Folin's reagent does not appear to be due to any of the other amino-acids known to be present. It is also probable that the same compound, tyrosine or other amino-acid, is the cause of the colour produced with Millon's reagent.

If the percentage of tyrosine is correct and if it is present as a constituent of another protein similar to ox muscle, the proportion of the latter in the gelatin would be about 10 %. This calculation however is based on too many assumptions to be of more than speculative interest.

Up to the present neither cystine nor any other compound containing sulphur has been isolated from gelatin though the presence of such unidentified compounds has been noted by Dakin [1920].

The gelatin used in these experiments after purification in the manner described above, contained 0·24 % of total sulphur, calculated on the dry substance. Krummacher [1903] after purifying gelatin by Kirchmann's method found 0·28 % S (of which 0·02 % was in the form of SO_3 and SO_4). The original commercial product used by Krummacher contained 0·62 % total S, of which 0·4 % was present as SO_3 and SO_4. Such an amount (0·24 %) of unoxidised sulphur would correspond with 0·9 % of cystine (or other compound containing a like proportion of S) and this can hardly be ascribed to impurities in the gelatin.

Results of the experiments on gelatin diets.

It was proposed to carry out three diet experiments in which low, medium and high amounts of gelatin nitrogen should be given in addition to the basal diet, in order to determine

(1) whether the minimum nitrogen loss on abundant nitrogen-free diet can be still further reduced by gelatin, and if so,

(2) what relation the amount of this reduction bears to the amount of gelatin ingested.

Two experiments were completed during the early part of 1921, but the

third had to be broken off through illness shortly after it was begun. It was repeated in September 1921 the conditions being somewhat modified on account of certain results that had in the meantime been obtained from other experiments carried out with Prof. C. J. Martin. These appeared to indicate that the amount of certain proteins required for nitrogen equilibrium could be greatly reduced if a carbohydrate diet very much in excess of the energy requirements was taken. In this last experiment, therefore, I increased the fuel value of the diet to the maximum that could be tolerated, so that the body weight was maintained and even increased during the first half of the period (see Fig. 1, Curve C). Synthesis of fat from the carbohydrate of the food was also indicated by the high respiratory quotient.

The diets for the three experiments are given together in Table IV. The diet taken during the experiment in which my minimum requirements were determined is also included for purposes of comparison.

Table IV.

	Nitrogen minimum 29/11/20—5/12/20 Total calories=2605 Calories per kilo=45[1] Calories supplied as Fat=31 %		Gelatin I 28/1/21—6/2/21 Total calories=2525 Calories per kilo=44 Calories supplied as Fat=6·4 %		Gelatin II 18/4/21—27/4/21 Total calories=2757 Calories per kilo=47 Calories supplied as Fat=6·5 %		Gelatin III 8/9/21—17/9/21 Total calories=3256 Calories per kilo=54 Calories supplied as Fat=0·86 %	
Food	Wt g.	N g.	Wt g.	N g.	Wt g.	N g.	Wt g.	N g.
Gelatin	—	—	66·67[2]	12·000	27·14[2]	4·885	41·89[2]	7·540
Corn starch	280	0·118	350	0·137	340	0·143	500	0·135
Dextrin	50	0·033	16	0·010	—	—	—	—
Butter and margarine	105	0·074	20	0·016	20	0·002	Cod oil 3	—
Honey	55	0·013	—	—	—	—	—	—
Sucrose	25	—	—	—	70	—	30	—
Lactose	65	0·008	180	0·023	250	0·032	300	0·039
Lemon juice	30 cc.	0·020	25	0·017	25	0·017	20	0·013
Vermouth	25 cc.	0·001	50	0·002	—	—	—	—
Tea	1200 cc.	0·072	350	0·028	250	0·020	600	0·043
Agar-agar	15	0·036	13	0·031	10	0·024	10	0·024
Salt mixture	5	—	10[3]	—	10[3]	—	10[3]	—
	—	0·375	—	12·264	—	5·123	—	7·794
Total fluid	—	—	2200–2500	—	2000	—	2200	—

[1] During the first five days of the experiment the fuel value of the diet was equal to about 52 cals. per kilo.

[2] Weight calculated as dry gelatin.

[3] Includes 4 g. sodium chloride.

No purification was attempted for experiment I. For II and III the gelatin, after purification by the method described in the text, was dissolved in hot water. Weighed amounts of this solution were transferred to bottles and sterilised in the autoclave. The figures for the nitrogen intake are based on a number of analyses of samples from different bottles.

Gelatin I (28th Jan. to 6th Feb. 1921).

During the three days previous to the experiment a mixed diet containing about 12 g. N was taken.

The experimental diet is given in Table IV. It included:

N in the form of gelatin ...	12·00 g.
N in accessories[1] (excluding tea)	0·23
Total N	12·23

[1] The nitrogen in the constituents of the basal diet.

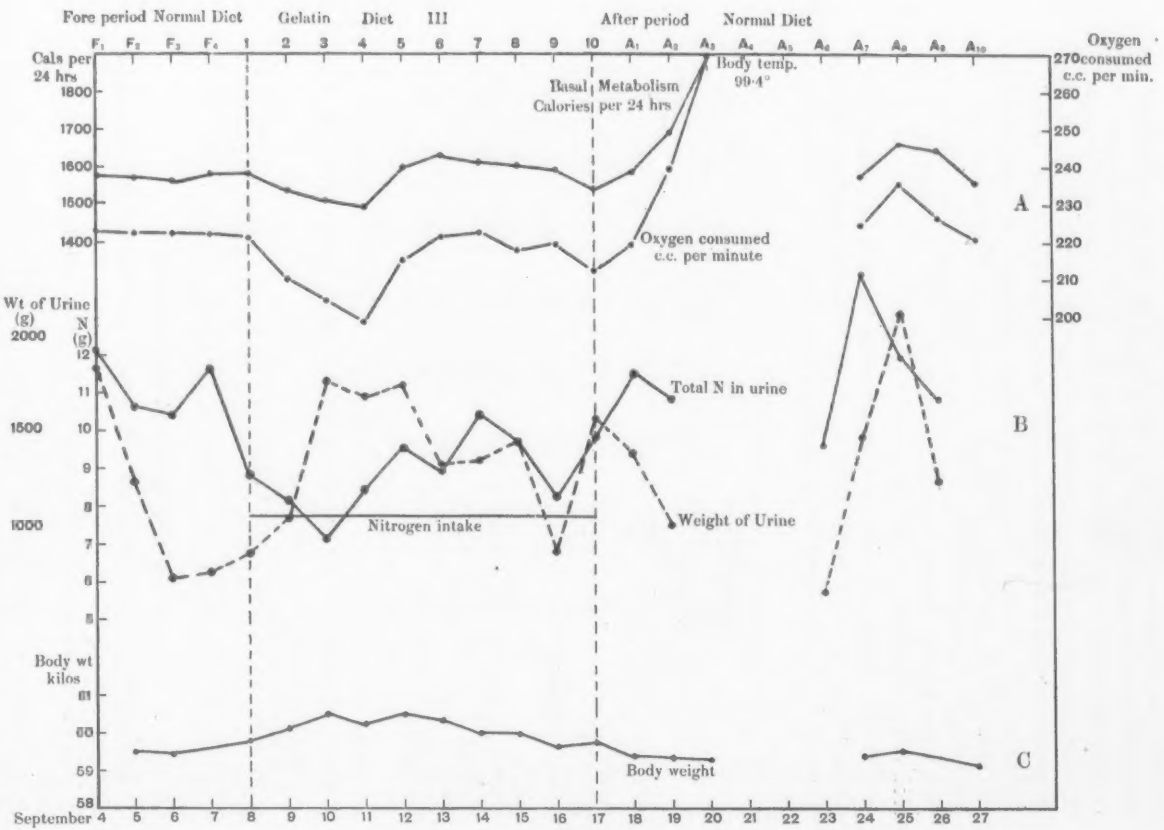
Fore period Normal Diet
Gelatin Diet III
After period Normal Diet
F1 F2 F3 F4 1 2 3 4 5 6 7 8 9 10 A1 A2 A3 A4 A5 A6 A7 A8 A9 A10
Cals per 24 hrs
1800
1700
1600
1500
1400
Basal Metabolism Calories per 24 hrs
Body temp. 99·4°
Oxygen consumed c.c. per minute
Oxygen consumed c.c. per min.
270
260
250
240
230
220
210
200
A
Wt of Urine (g)
2000
1500
1000
N (g)
12
11
10
9
8
7
6
5
Total N in urine
Nitrogen intake
Weight of Urine
B
Body wt kilos
61
60
59
58
Body weight
C
September 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27

Fig. 1.

A few nitrogen-free biscuits made from starch, dextrin and agar were chewed at each meal to increase the flow of saliva. The rest of the starch etc. was taken in the raw state as already described.

The results of the experiments are shown in Table V. It will be seen that the nitrogen output in the urine remained almost stationary for three days, then rose and a series of oscillations set in.

Table V.

Date	Day of exp.	Body weight k.	Weight of urine g.	Sp. gr. of urine g.	Creatinine N g.	Total N in urine[1] g.	N in faeces g.	Total N (U + F) g.	Balance
Jan. 28	1	58·75	1447	1·0175	0·44	12·37	(1·38)[2]	(13·75)	(−1·50)
29	2	—	1790	1·014	0·53	12·42	1·38	13·80	−1·57
30	3	—	714	1·023	0·46	12·38	1·38	13·76	−1·53
31	4	—	1236	1·015	0·48	14·05	1·38	15·43	−3·20
Feb. 1	5	—	1550	1·013	0·52	12·84	1·38	14·22	−1·99
2	6	—	1496	1·015	0·53	14·42	1·38	15·80	−3·57
3	7	57·80	1200	1·016	0·51	13·13	1·38	14·51	−2·28
4	8	—	1300	1·015	0·53	14·20	1·38	15·58	−3·35
5	9	—	1601	1·014	0·50	12·27	1·38	13·65	−1·42
6	10	57·55	1914	1·014	0·54	14·44	1·38	15·82	−3·59
Average for whole period					0·50	13·25	1·38	14·63	−2·40
Average for the last six days					0·52	13·55	1·38	14·93	−2·70

[1] The "caffeine N" = 0·03 g. (from tea) has been subtracted from the total urinary nitrogen.

[2] The faeces were only collected for the last nine days.

Gelatin II (15th–27th April 1921).

During the two days previous to this experiment the diet consisted of eggs, milk, bread, potatoes, butter and apples, and contained 11·2 g. nitrogen. The experimental period was divided into two parts.

During the first three days the basal diet was supplemented by 250 g. of egg-white while during the last ten days this was replaced by gelatin. The two diets contained:

	Three days 15th–17th April	Ten days 18th–27th April
N in form of egg-white ...	5·0	—
„ gelatin ...	—	4·88
N in accessories (excluding tea)	·21	·22
Total N	5·21	5·10
Fuel value	2580 cals = 44 cals per kilo	2757 cals = 47 cals per kilo (6·5 % supplied by fat)

The results are shown in Table VI.

A pronounced negative balance occurred on the egg-white diet and the nitrogen output during the first few days of the gelatin period is slightly above the average of the last six days.

Gelatin III (8th–17th Sept. 1921).

During the preceding four days a mixed diet containing about 12 g. N and equal to about 2400 calories was taken. On the 7th Sept. 100 g. lactose and 100 g. starch were consumed in addition to the above, making the total calories 3130.

Table VI.

Date	Day of exp.	Body weight k.	Weight of urine g.	Sp. gr. of urine g.	Creatinine N g.	Total N in urine[1] g.	N in faeces g.	Total N (U+F) g.	Balance
First period:									
April 15	*E* 1	59·3	1351	1·017	—	7·30	—	—	—
16	*E* 2	—	1155	1·019	—	5·91	—	—	—
17	*E* 3	—	962	1·019	—	6·09	—	—	—
Second period:									
April 18	1	58·85	1517	1·013	0·52	7·42	1·28	8·70	−3·60
19	2	—	1779	1·012	0·56	6·32	1·28	7·60	−2·50
20	3	—	904	1·018	0·55	6·85	1·28	8·13	−3·03
21	4	—	1125	1·015	0·53	6·73	1·28	8·01	−2·91
22	5	—	833	1·019	0·55	5·68	1·28	6·96	−1·86
23	6	—	1148	1·017	0·54	7·12	1·28	8·40	−3·30
24	7	—	1523	1·013	0·54	6·18	1·28	7·46	−2·36
25	8	58·35	817	1·021	0·53	6·12	1·28	7·40	−2·30
26	9	—	1209	1·015	0·52	6·77	1·28	8·05	−2·95
27	10	—	1020	1·017	0·53	6·56	1·28	7·84	−2·74
28	—	58·15	—	—	—	—	—	—	—
Average of whole period				... ...	0·54	6·57	1·28	7·85	−2·75
Average of last six days				... ...	0·53	6·41	1·28	7·69	−2·59

[1] The "caffeine N" = ·02 g. (from tea) has been subtracted from the total urinary nitrogen.

The experimental diet is given in Table IV. It included:

N in the form of gelatin	7·54 g.
N in accessories (excluding tea)	·21
Total N	7·75

From Sept. 14th the fuel value was increased by 100 calories taken in the form of starch biscuits and honey. The increased nitrogen is negligible.

The daily exercise consisted of a walk of from five to seven miles.

The results are shown in Table VII.

Here as in Gelatin I the nitrogen output during the first four days is below the average for the whole period.

Table VII.

Date	Day of exp.	Body weight k.	Weight of urine g.	Sp. gr. of urine g.	Creatinine N g.	Total N in urine[1] g.	N in faeces g.	Total N (U+F) g.	Balance
Sept. 8	1	59·8	850	1·0225	0·59	8·77	1·54	10·31	−2·56
9	2	60·1	1038	1·0175	0·59	8·12	1·54	9·66	−1·91
10	3	60·5	1764	1·011	0·60	7·11	1·54	8·65	−0·90
11	4	60·25	1680	1·013	0·58	8·43	1·54	9·97	−2·22
12	5	60·5	1735	1·013	0·60	9·49	1·54	11·03	−3·28
13	6	60·35	1320	1·0165	0·59	8·87	1·54	10·41	−2·66
14	7	60·0	1347	1·0155	0·57	10·37	1·54	11·91	−4·16
15	8	59·93	1439	1·015	0·59	9·68	1·54	11·22	−3·47
16	9	59·65	859	1·0215	0·59	8·20	1·54	9·74	−1·99
17	10	59·78	1555	1·0145	0·60	9·84	1·54	11·38	−3·63
Average for the whole period				... ...	0·59	8·89	1·54	10·43	−2·68
Average for the last six days				... ...	0·59	9·41	1·54	10·95	−3·20

[1] The "caffeine nitrogen" = ·04 g. (from tea) has been subtracted from the total urinary nitrogen.

Basal metabolism.

My basal metabolism was determined each day while on the experimental diet and during the periods immediately before and after.

The method adopted was that of the Douglas bag, the expired air being analysed in Haldane's gas analysis apparatus. From the 4th to the 7th of September the estimation was made at 9 a.m., fasting, after a walk of one mile followed by a resting period of at least 30 minutes. All the remaining determinations were made between 7.30 a.m. and 8 a.m. on waking. The results are shown in Table VIII.

A slight decrease in the basal metabolism occurred at the commencement of the experimental diet but the normal level was regained by about the fourth day. This decrease coincides with the lower nitrogen output, as may be seen in Fig. 1 A and B, but it is not possible to say whether the two are in any way connected. No corresponding decrease occurred in the creatinine output which was constant throughout the period.

In calculating the basal metabolism in calories per 24 hours the protein oxidation has been ignored but the error thus introduced is less than + 1 %. For those days in which the R.Q. is greater than 1 a correction has been made for the nett heat production due to synthesis of fat from carbohydrate by adding 0·3 of the calories equivalent to the excess of the CO_2 output over the oxygen consumed. I am aware that this is an arbitrary estimate, but the possible error involved is only slight.

Table VIII.

Date	Day of exp.	Diet during previous 24 hours (calories per kilo)	Oxygen consumed per minute (cc)	R.Q.	Calories per 24 hours
Sept. 4	*F* 1	Normal (40)	223·0	0·831	1575
5	*F* 2	,, ,,	222·5	0·830	1571
6	*F* 3	,, ,,	222·4	0·803	1561
7	*F* 4	,,	222·3	0·866	1580
8	*G* 1	Normal + 200 g. starch (54) lactose mixture	221·2	0·904	1582
9	*G* 2	Carbohydrate + gelatin (54)	210·1	1·012	1537
10	*G* 3	,, ,,	204·8	1·043	1510
11	*G* 4	,, ,,	199·0	1·093	1492
12	*G* 5	,, ,,	215·3	1·065	1600
13	*G* 6	,, ,,	221·6	1·051	1633
14	*G* 7	,, ,,	222·8	0·967	1614
15	*G* 8	,, ,,	218·0	1·040	1604
16	*G* 9	,, ,,	219·8	0·967	1592
17	*G* 10	,, ,,	212·9	0·968	1542
18	*A* 1	,, ,,	218·7	0·979	1587
19	*A* 2	Normal diet	239·8	0·823	1691
20	*A* 3	,,	269·3[1]	0·805	1893
24	*A* 7	,,	224·3	0·801	1574
25	*A* 8	,,	235·5	0·809	1660
26	*A* 9	,,	226·3	0·823	1596
27	*A* 10	,,	220·9	0·830	1559

[1] The body temperature was 99·4° when this determination was made. Several attacks of vomiting had occurred during the previous night. The condition became worse and necessitated some days' rest.

The normal *basal metabolism* calculated from Harris and Benedict's formula for a man of age 37, weight 59·8 kilo, height 173·5 cm. would be $66{\cdot}4730 + 13{\cdot}7516 \times 59{\cdot}8 + 5{\cdot}0033 \times 173{\cdot}5 - 6{\cdot}755 \times 37 = 1506$ calories.

General Considerations.

My usual mode of life was followed throughout these experiments, eight or nine hours of each day being occupied with laboratory work. The only form of exercise was a walk of from two to seven miles. No great difficulty was found in consuming the diet although a feeling of nausea was frequently experienced. This was greatly intensified by the excessive quantity of food in the third experiment; the tongue became furred and more or less headache was common. Slight diarrhoea occurred in all three periods, faeces of fluid consistency being passed two or three times a day. The diet was however very well assimilated scarcely any starch being found in the faeces. Traces of reducing sugar were regularly present in the urine during the third experiment but only occasionally during the first two.

It was intended that a period on nitrogen-free diet should follow immediately on the third gelatin diet in order to determine my minimum requirements once more. This was not possible, and indeed much difficulty was experienced in completing the ten days proposed for the above experiment.

In the two experiments (*G* I, *G* III) which followed a normal mixed diet the average nitrogen output during the first four days was lower than that for the remainder of the period while the reverse of this was observed when the previous diet had been insufficient to satisfy the protein requirements (*G* II) and a considerable negative balance had occurred. These differences are probably due to the influence of the protein of the preceding diet, a diminishing store of which, perhaps in the form of amino-acids, remains in the body for some days. The first four days have therefore been excluded in considering the results. Another disturbing factor is the variation in the urine nitrogen from day to day. This frequently amounted to more than 20 % of the average output and showed no relationship whatever to the volume of the urine. Consequently it cannot be explained by diuresis.

Summary and Discussion of Results.

The results obtained in the three experiments are summarised in Table IX, the average figures being given for the last six days of each ten day period. The average amounts of the nitrogen intake and output on the last three days of the earlier experiment on "nitrogen-free" diet are also included.

Table IX

Date	Body weight (kilos)	Fuel value of diet, cals per kilo	Nitrogen intake		Nitrogen output				Balance
			Gelatin	Accessories	Urine	Faeces	Total	Creatinine	
			g.	g.	g.	g.	g.	g.	
3. xii. 20–5. xii. 20	58·0	52–44	—	·30	2·06	1·13	3·19	0·49	−2·89
1. ii. 21–6. ii. 21	57·8	44	12·00	·23	13·55	1·38	14·93	0·52	−2·70
21. iv. 21–27. iv. 21	58·4	47	4·88	·22	6·41	1·28	7·69	0·53	−2·59
11. ix. 21–17. ix. 21	60·2	54	7·54	·21	9·41	1·54	10·95	0·59	−3·20

In attempting to calculate the amount of body protein spared by the gelatin from these results we are met by two difficulties, namely what is to be done with that part of the intake due to the nitrogen of the accessories, and with that part of the nitrogen output due to the faeces.

Nitrogen of the accessories. The nitrogen from the tea does not appear in the table, having been subtracted both from intake and output. The assumption that this is all "caffeine N" is not strictly correct but as the total amount is very small, usually under 0·05 g. any error involved in this mode of treatment must be negligible. The greater part of the accessory nitrogen comes from the corn starch. In McCollum's account of his experiments on pigs, no mention is made of any nitrogen arising from this source although large quantities (1700 g.?) of corn starch were given. If this starch contained as much nitrogen as the samples used by me the nitrogen intake from this source may easily have been 0·5 g. or more. We do not know in what form this nitrogen is present nor its value in the human body and cannot therefore estimate its effect on the nitrogen output. If the amount and nature of such accessories are the same during the determination of the nitrogen minimum and experiments with gelatin, the following argument might be applied. If the value of this nitrogen in the accessories is zero then the real nitrogen requirements will be less than the observed output by the full amount of such nitrogen intake since the latter must be excreted in addition to the nitrogen resulting from the protein metabolism of the body. If the value of this nitrogen for the replacement of body nitrogen is 100 % then it will spare an equal amount of the latter and the observed output on the so-called "nitrogen-free" diet will represent the actual minimum requirements. But in this case an equal amount of gelatin will also be spared when gelatin is taken, and the apparent sparing effect of the gelatin will thus be increased by the same amount.

In either case the real saving of body nitrogen due to the gelatin will be less than the apparent saving, *i.e.* the difference between the negative balance on the gelatin and the minimum nitrogen output on the "nitrogen-free" diet, by an amount equal to the nitrogen of the accessories. This will also hold for all values of the latter between 0 and 100 %. Unfortunately the proviso that the accessories should be the same on both diets does not strictly hold in the above experiments but if the butter nitrogen be subtracted from the total intake on the "nitrogen-free" diet the remainder is practically the same as the accessory nitrogen on the gelatin diets. The butter nitrogen would probably have a high value and I have therefore assumed that it does not appreciably increase the nitrogen output. The rest of the accessory nitrogen (·22 g. average) has been deducted in calculating the amount of body nitrogen saved by the gelatin. This is not strictly accurate but is probably the best that can be done with the figures. The above argument however ignores the possibility of complementary action of the accessories and the gelatin. It has been already pointed out that such action occurs when gelatin is fed with certain cereal proteins. McCollum, Simmonds and Pitz [1917] have shown that a mixture of oat protein and gelatin has a higher value than either alone or than oat protein plus caseinogen. It is impossible to say whether the results in my experiments were affected by such complementary action but if this did occur the real value of the gelatin alone is still less than the calculations appear to show.

Faecal nitrogen. The problem of how to treat the nitrogen excreted in the faeces is even more difficult. In McCollum's experiments the urinary nitrogen is alone considered, that in the faeces being estimated merely as a check on the complete absorption of the food protein. He considers that the nitrogen of the urine represents the essential tissue metabolism, while that of the faeces represents losses which may be termed accidental in character.

It has been shown by Rubner [1919] that the increased amount of nitrogen in the faeces of men, when fed on various diets, above that found on a carbohydrate diet cannot be taken as entirely due to undigested food protein, but that a considerable proportion of the increase comes from the body. In my experiments somewhat large variations were observed in the nitrogen of the faeces and these may have been due to the slight diarrhoea which occurred. Probably most of the nitrogen came from the body but the possibility that a small amount of gelatin escaped absorption must not be overlooked. We know very little about the relationship between loss of body nitrogen through the intestines and that excreted in the urine, but there seems to be no evidence that an increase in the former is accompanied by a decrease in the latter. The reverse of this is perhaps more probable.

This question remains at present the limiting factor for the accuracy of such experiments. I have attempted to define the limits between which the true conclusion from my results is to be found, by calculating the amount of body nitrogen saved by the gelatin in two ways. In the first (A) I have assumed that my minimum nitrogen requirements are represented by the sum of the nitrogen in the urine and that in the faeces on the nitrogen-free diet, and that the difference between the latter amount and the corresponding excretion on the gelatin diets represents unabsorbed gelatin nitrogen.

Table X.

	N Intake			A			B		
					Body Nitrogen saved			Body Nitrogen saved	
Experiment	Gelatin g.	Accessories g.	N Balance g.	N minimum g.	g.	% of minimum	N minimum g.	g.	% of minimum
R.R. II	4·88	0·22	−2·59	3·19	0·38	11·9	3·34	0·53	15·9
R.R. III	7·54	0·21	−3·20	3·19	0	0	3·60	0·19	5·3
R.R. I	12·00	0·23	−2·70	3·19	0·26	8·1	3·44	0·51	14·7
McCollum's pig average of last six day	2·62	?	−2·35	3·68	1·33	36·1	3·66	1·31	36·3

In the second (B) I have assumed that the gelatin is completely absorbed and that the minimum requirements for such periods are represented by the output in the urine on "nitrogen-free" diet plus the nitrogen in the faeces on the gelatin diet under consideration. The truth probably lies somewhere between these two extremes. The results of these calculations are given in Table X. McCollum's figures are also included for comparison. The accessory nitrogen has been in each case deducted from the apparent amount of body nitrogen saved.

The maximum saving in terms of the nitrogen minimum is thus 11·9 % if the first method of calculation is employed and 15·9 % if the second is used.

The fact that this was obtained with the lowest amount of gelatin would appear to prove that the effect is not due to any impurity, in which case there should be a proportionality between the amount saved and the gelatin intake. In this connection however the possibility that the increased protein in the diet entails an increased loss of body nitrogen must not be overlooked, although the creatinine excretion does not lend any support to such an hypothesis.

The results of the first and second experiments agree well between themselves, but in the third a much lower value was apparently indicated. It will be noticed however that the creatinine excretion in the experiment was higher than the normal and the body weight was also higher. This nitrogen minimum may therefore have been higher than the amount shown (perhaps owing to the excessive amount of food taken) in which case the calculated result would be too low.

All these values are much lower than those found by McCollum and this discrepancy cannot be explained except by assuming a difference in the metabolism of man and pig. If the values are calculated in terms of the urinary output alone they are all proportionately increased—the value for my experiment *G* II then becoming 25·7 % of the minimum, but the difference between the man and the pig still persists.

The creatinine output has been shown to bear some close relationship with the minimum nitrogen output. I therefore attempted to compare my results with McCollum's in terms of the amounts of creatinine excreted in the several experiments. I ignored the faeces and calculated the negative balance by subtracting the nitrogen intake from the output in the urine. The ratio of this balance to the average amount of creatinine nitrogen excreted during the same period is shown in the last column of Table XI. The last six days of each period have been alone considered.

Table XI.

Experiment	Nitrogen intake	Nitrogen in urine	Nitrogen balance	Creatinine N	Nitrogen balance / Creatinine nitrogen
	g.	g.	g.	g.	
R.R. II	5·10	6·41	−1·31	0·53	2·47
R.R. III	7·75	9·41	−1·66	0·59	2·81
R.R. I	12·23	13·55	−1·32	0·52	2·54
McCollum's pig	2·62	3·89	−1·27	0·48	2·65

There is obviously no discrepancy between our results when they are considered in this way, though what this agreement implies is not easy to state. It has been shown in many papers by Grafe (1912–1914), Abderhalden [1915], Underhill and Goldschmidt [1913] and others, that many nitrogen compounds other than amino-acids, namely organic ammonium salts, urea etc., have the capacity to spare a certain proportion of the loss of body nitrogen occurring on a carbohydrate diet. The results obtained by these workers do not agree in all points but the amount of nitrogen thus spared appears to be of the same order as that spared with gelatin in my experiments. It may well be that the action of the gelatin is of the same nature as that of these simpler

compounds and consists essentially in the reduction of the waste of amino-acids derived from body protein through deaminisation and subsequent oxidation in the body. The amino-acids of the gelatin and the ammonia and urea produced from them can play a part in the reversible reactions that are constantly proceeding in the body and thus influence the resulting equilibrium.

$$\text{Amino-acid} \rightleftarrows \underset{\text{(Ketonic or hydroxy acid)}}{\text{Non-nitrogenous compound}} + NH_3 \rightleftarrows \text{urea.}$$

If this is true the loss of body nitrogen when both carbohydrate and gelatin are fed may represent a "N-minimum" that corresponds more closely with the specific nitrogen requirements of the body than does the output on carbohydrate diet alone. This may perhaps be the explanation of the close agreement between the ratios of such loss to the creatinine nitrogen shown by my experiments and those of McCollum.

In conclusion I would express my very sincere thanks to Prof. C. J. Martin for his constant encouragement and advice throughout this investigation.

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XVII. DISTRIBUTION OF THE NITROGENOUS CONSTITUENTS OF THE URINE ON LOW NITROGEN DIETS.

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During the course of experiments carried out in conjunction with Prof. C. J. Martin on ourselves, with the object of determining our minimum nitrogen requirements and the biological value of certain proteins, the opportunity was taken of investigating the distribution of the nitrogenous constituents in the urine when the total nitrogen output had reached a very low level.

In the particular experiments referred to in this paper, a diet consisting of carbohydrate and fat (corn starch, sucrose, lactose, honey, butter) together with a little lemon juice, inorganic salts and agar-agar was taken during seven days. The fuel value of this diet was equal to 44–52 calories per kilo body weight and the nitrogen content was about 0·3 g. A little tea and coffee was also taken but the nitrogen in these beverages was assumed to be "caffeine nitrogen" and to be excreted as such in the urine during the same 24 hours. It was therefore subtracted from the total nitrogen before calculating the percentage amounts of the other constituents. The above assumption is of course not strictly accurate, but in the one experiment (R.R.) the total amount of such nitrogen is very small, less than 0·1 g., so that any error thus introduced may be considered negligible.

The period on this low nitrogen diet was immediately followed by another of five days during which the same basal diet with the addition of a little milk was taken, the nitrogen intake being thus raised to about 3 g.

The total nitrogen in the urine and in all constituents of the diet was estimated by Kjeldahl's method, urea by van Slyke's urease method, ammonia by Folin's aeration method, amino-acids by formal titration after removal of the ammonia, creatinine by the method of Folin, and uric acid by that of Hopkins as modified by Folin and Schaffer.

The results are set out in Table I. In Table II the distribution of nitrogen on the days for which the total nitrogen output reached its lowest values has been compared with corresponding figures obtained by other investigators.

It will be seen that these observations are in complete agreement with Folin's [1905, 2] generalisations respecting the variation in the distribution of the urinary nitrogen. The creatinine nitrogen is practically constant and is

Table I.

Subject: C.J.M. Weight Nov. 28th—61·9 kilos; Dec. 5th—60·5 kilos

Date 1920 Dec.	Day of exp.	Intake N g.	Volume of urine cc. Sp. gr. of urine	Total N g.	Total N less caffeine N g.	Urea N g.	Ammonia N g.	Amino-acid N g.	Creatinine N g.	Uric acid N g.	Undetermined N g.	In percentage of total N (less caffeine N) Urea	Ammonia	Urea + ammonia	Amino-acid	Creatinine	Uric acid	Undetermined
4	6	·35 } ·29[1] }	1421 1·014	2·69	2·40	1·27	·29	·05	·45	·07	·27	52·9	12·1	65·0	2·1	18·7	2·9	11·3
5	7	·33 } ·26[1] }	908 1·020	2·39	2·13	1·08	·21	·05	·43	·07	·29	50·7	9·9	60·6	2·3	20·2	3·3	13·6
6[2]	8	3·37 } ·25[1] }	1708 1·011	2·67	2·42	1·24	·30	·11	·47	·02	·28	51·2	12·4	63·6	4·5	19·4	·8	11·5
7	9	3·36 } ·17[1] }	1207 1·0165	2·74	2·57	1·39	·23	·08	·42	·06	·39	54·1	9·0	63·1	3·1	16·3	2·3	15·2
8	10	3·42 } ·29[1] }	1060 1·020	3·30	3·01	1·91	·24	·06	·45	·06	·29	63·4	8·0	71·4	2·0	15·0	2·0	9·6
9	11	3·40 } ·34[1] }	1128 1·0185	3·46	3·12	1·96	·28	·06	·44	·08	·30	62·8	9·0	71·8	1·9	14·1	2·6	9·6
Subject: R.R. Weight Nov. 28th—58·6 kilos; Dec. 5th—57·7 kilos.																		
3	5	·32 } ·09[1] }	1886 1·007	2·08	1·99	·74	·35	·04	·50	·07	·29	37·2	17·6	54·8	2·1	25·1	3·5	14·5
4	6	·30 } ·07[1] }	1703 1·010	2·24	2·17	·98	·27	·05	·50	·10	·27	45·2	12·4	57·6	2·3	23·0	4·6	12·4
5	7	·27 } ·06[1] }	1547 1·009	2·07	2·01	·91	·25	·04	·47	·07	·27	45·3	12·4	57·7	2·0	23·3	3·5	13·4
6[2]	8	2·98 } ·10[1] }	1783 1·010	2·59	2·49	1·26	·31	·04	·50	·07	·31	50·6	12·5	63·1	1·6	20·1	2·8	12·4
7	9	2·94 } ·09[1] }	1991 1·011	3·04	2·95	1·71	·29	·04	·50	·03	·38	57·9	9·8	67·7	1·4	17·0	1·0	12·9
8	10	3·09 } ·16[1] }	1403 1·012	3·38	3·22	2·06	·26	·04	·45	·05	·36	64·0	8·1	72·1	1·2	14·0	1·5	11·2
9	11	2·91 } ·15[1] }	1134 1·014	3·01	2·86	1·69	·29	·04	·45	·09	·30	59·1	10·1	69·2	1·4	15·8	3·1	10·5
10	12	2·97 } ·18[1] }	1260 1·015	3·01	2·83	1·89	·26	·03	·46	·02	·17	66·8	9·2	76·0	1·1	16·2	·7	6·0

[1] N from tea and coffee—taken as "caffeine N." [2] Milk diet began on Dec. 6.

Table II.

Observer	Subject	Body weight	Day of exp.	Intake N g.	Total N g.	Urea N g.	Ammonia N g.	Amino-acid N g.	Creatinine N g.	Uric acid N g.	Undetermined N g.	Percentages of total N Urea	Ammonia	Urea + Ammonia	Amino-acid	Creatinine	Uric acid	Undetermined
Folin [1905, 1]	H.B.H.	85·7	7	1	3·6	2·2	·42	—	·60	·09	·27	61·7	11·3	73·0	—	17·2	2·5	7·3
,,	E.S.A.	55·7	7	1	2·8	1·7	·28	—	·41	·08	·32	60·7	10·0	70·7	—	14·0	3·0	11·6
,,	A.H.	70·7	7	1	3·5	2·1	·27	—	·50	·14	·52	59·3	7·8	67·1	—	14·1	4·0	14·8
Zeller [1914]	—	67·9	17	1·04	2·88	1·42	·34	·22	·63	·15	·05	49·3	11·7	61·0	7·5	21·9	5·1	1·8
Graham and Poulton [1912]	G.G.	61·4	9	·91	2·25	1·047	·343	—	·598	·072	·19	46·54	15·25	61·79	—	26·58	3·2	8·44
Robison	C.J.M.	60·5	7	·33 ·26[1]	2·13[2]	1·08	·21	·05	·43	·07	·29	50·7	9·9	60·6	2·3	20·2	3·3	13·6
,,	R.R.	57·8	5	·32 ·09[1]	1·99[2]	·74	·35	·04	·50	·07	·29	37·2	17·6	54·8	2·1	25·1	3·5	14·5
,,	R.R.	—	7	·27 ·06[1]	2·01[2]	·91	·25	·04	·47	·07	·27	45·3	12·4	57·7	2·0	23·3	3·6	13·4

[1] N from tea and coffee—taken as "caffeine N." [2] Total urine N less "caffeine N."

equal to 7·3 mg. (C.J.M.) and 8·2 mg. (R.R.) per kilo body weight. These amounts like those of the other constituents are very similar to those found by Folin and others. The values for the total nitrogen are amongst the lowest on record and correspond with still further reductions in the percentage of urea nitrogen, this falling in one instance to 37 % of the total. The last figure was however accompanied by a somewhat high percentage of ammonia, but the sum of the urea and ammonia nitrogen was only 54·6 % of the whole amount. It would have been interesting to discover whether this sum (urea + ammonia) could have been further reduced by the ingestion of alkalies or whether any decrease in the ammonia nitrogen would have been accompanied by an increase in the urea.

The question as to whether any part of the urea nitrogen represents what Folin has termed the endogenous metabolism remains open.

I wish to express my indebtedness to Prof. C. J. Martin for his interest and help in this work.

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XVIII. THE ESTIMATION OF TOTAL SULPHUR IN URINE.

By ROBERT ROBISON.

From the Lister Institute.

(*Received January 27th, 1922.*)

The methods for the estimation of total sulphur in urine are less satisfactory than those available for determining many of the other urinary constituents. A high degree of accuracy is specially important when we are concerned with the amount of unoxidised or "neutral" sulphur in the urine. This can only be determined by subtracting the amount of sulphates from that of the total sulphur and any error in the latter value falls on the relatively small difference.

Benedict's [1909] method in which the oxidation is effected with a mixture of copper nitrate and potassium chlorate has been criticised on the ground that considerable losses frequently occur through spattering during the initial stages of the reaction. Denis [1910] has stated that out of forty analyses mechanical loss resulted in every case. She has proposed to substitute a solution of copper nitrate, ammonium nitrate and sodium chloride in place of Benedict's oxidising reagent, thereby reducing the vigour of the reaction.

In my hands Denis' modification has not proved so reliable as the original method. Decrepitation is certainly less troublesome but the results are frequently too high, the errors amounting at times to more than 10 mg. The following series taken from nearly a hundred analyses are fairly representative:

25 cc. urine (1) gave 0·0711, 0·0719, 0·0783, 0·0826 g. $BaSO_4$,

„ „ (2) „ 0·0864, 0·0901, 0·0865, 0·0899, 0·0877 g. $BaSO_4$.

The residue, even after prolonged ignition always contained appreciable amounts of nitrate, this being probably due to sodium nitrate formed from the sodium chloride and copper nitrate since the latter is readily decomposed at temperatures lower than those used. Whether the presence of this nitrate would alone account for the high results I am not able to say. Considerable errors in this direction were observed by Kolthoff and Vogelensang [1919] using higher concentrations of both sulphate and nitrate.

Consistent results were usually obtained by Benedict's original method, using an electric hot plate as suggested by Givens [1917] although in spite of this precaution vigorous spattering sometimes occurred. Only slight traces of nitrate were found in the residues after ignition and it is probable that these arose from the small amounts of alkali salts in the reagent and in the urine itself. The necessity of igniting the residue at a red heat was however

a frequent cause of spoiled analyses through the cracking of the porcelain basins. Probably those used by Benedict were of better quality than are at present obtainable.

Errors due to the use of coal gas for igniting the residue.

Neither Benedict nor Denis appears to have recognised the possibility of errors from the presence of sulphur in the coal gas used for the ignition of the residue. Folin [1903] advised spirit burners for his original method and according to Liebesny [1920] errors of 8–10 % in the amount of neutral sulphur may arise from the use of gas. My own experience confirms this. In determining the blank of the oxidising solution referred to below, 2·5 cc. was found to yield 0·7 mg. $BaSO_4$, using a spirit burner, and 2·4 mg. using an argand burner with coal gas. If this error were constant in amount it would be cancelled in subtracting the blank but such was not found to be the case. Probably the absorption of oxides of sulphur depends on the surface area of the residue as well as on the total quantity present in the gas.

The use of a spirit burner is therefore essential if accurate results are to be obtained.

Since copper nitrate, which forms the basis of Benedict's reagent, is decomposed at comparatively low temperatures it seemed possible that the necessity of igniting the residue at a red heat might be avoided if neither chlorate nor alkali salts were introduced. Other substances were therefore tried as a diluent for the copper nitrate and of these copper chloride was found to be the most satisfactory. In the presence of a suitable proportion of this salt spattering never occurs, while the nitrate can be completely decomposed by heating the residue over a small spirit stove of the simplest kind. The temperature of a good argand burner is sufficiently high but gas cannot be used.

The oxidising reagent finally adopted has the following composition:

Copper nitrate (cryst.)	40 g.
Copper chloride (cryst.)	15 g.
Water	to 100 cc.

2·5 cc. of this solution are added to 10 cc. of the urine in a 4-inch porcelain basin and evaporated to dryness on a water-bath or electric hot plate. The oxidation can be started on the hot plate, or over a very small spirit flame. It proceeds rapidly but smoothly, leaving a coherent residue which frequently swells up. The dish is then heated over a broad spirit flame for 20 minutes. A spirit stove of the common kind is suitable but a sound tin, half filled with methylated spirit answers very well. A better flame is obtained if a number of holes are punched about half-way up the tin. The residue is dissolved in 10 cc. of 2*N* HCl and diluted with 300 cc. distilled water. The sulphate is precipitated in the boiling solution with 10 cc. of a 5 % solution of barium chloride, dropped in very slowly by means of a dropping tube. The precipitate is allowed to stand overnight before being filtered.

In the following analyses the precipitates were weighed in platinum crucibles.

For 25 cc. urine, 6·25 cc. of the oxidising solution, 20 cc. 2*N* HCl, 400 cc. H_2O and 15 cc.–20 cc. of 5 % barium chloride solution were used. A blank determination must be carried out with the reagents.

Two series, each of eight estimations, of the total sulphur in a normal urine yielded the following results.

	$BaSO_4$ from 10 cc. urine		$BaSO_4$ from 25 cc. urine
1.	0·0451	1.	0·1140
2.	0·0454	2.	0·1143
3.	0·0460	3.	0·1135
4.	0·0454	4.	0·1141
5.	0·0456	5.	0·1138
6.	0·0457	6.	0·1133
7.	0·0461	7.	0·1141
8.	0·0454	8.	0·1148
	Average 0·0456 g.		Average 0·1140 g. equivalent to 0·0456 g. for 10 cc.

It is doubtful whether any gain in accuracy is achieved by the use of 25 cc. instead of 10 cc. of urine.

In all the above estimations the filtrates were examined for nitrates by the very sensitive diphenylamine test, slight traces being found in one or two cases only.

The accuracy of the method was checked by estimating the total sulphur in two solutions of cystine containing also 2 % of urea and a definite volume of *N*/10 H_2SO_4 neutralised by an equal amount of *N*/10 NaOH.

The sample of cystine, for which I am indebted to the kindness of Dr J. C. Drummond, was analysed by the Carius method:

0·1878 g. gave 0·3679 $BaSO_4$; calculated 0·3681 g. $BaSO_4$.

$BaSO_4$ from 10 cc. solution A	$BaSO_4$ from 10 cc. solution B
0·0671	0·0662
0·0670	0·0659
0·0675	0·0661
0·0675	0·0659
—	0·0659
—	0·0663
0·0673 g.	0·0661 g.
Calculated 0·0678 of which 0·0212 is from cystine.	Calculated 0·0668 of which 0·0202 is from cystine.

This method has been employed for some time by myself and others in connection with metabolism experiments in this laboratory and has proved satisfactory.

I wish to thank Miss M. Tazelaar and Miss M. H. Carr for much valuable help in carrying out the numerous analyses, only a very small proportion of which have been recorded above.

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XIX. THE ACTION OF YEAST-GROWTH STIMULANT.

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(*Received January 30th, 1922.*)

Williams [1919] and Bachman [1919] have confirmed the observation of Wildiers [1901] that certain yeasts are only able to grow at the expense of ammonium salts provided that a heavy inoculation is employed or a small amount of organic material (termed "bios" by Wildiers) is added to the medium. After a study of the ability of various substances to produce this effect, they suggest that the essential substance, which we will continue to call "bios" for the sake of convenience, is identical with the water-soluble *B* or anti-beri-beri vitamin. Williams [1920] has gone further, and has elaborated a method for measuring quantitatively the amount of anti-beri-beri vitamin present in any substance by observing its effect on the growth of yeast.

This suggestion has given rise to much discussion [see Eddy, 1921] and at present it is generally considered that the case for the identity of the two principles is not proven.

Lemon juice freed from citric acid by the method of Harden and Zilva [1918] when added to a mineral nutrient solution in small quantities enables a yeast to grow which could not grow in its absence. As animal feeding experiments show that the amount of water soluble *B* vitamin in lemon juice is very small relative to that in yeast extract [see Osborne and Mendel, 1920], it was thought that an investigation of its effect on the growth of yeast in mineral nutrient solutions might throw some light on the way in which the stimulation is brought about.

Preliminary experiments were performed to ascertain the smallest amount of lemon juice capable of producing growth in a nutrient solution made up as follows (Williams):

Saccharose	...	20 g.
$(NH_4)_2SO_4$	...	3 g.
KH_2PO_4 ...	...	2 g.
$CaCl_2$ ...	...	0·25 g.
$MgSO_4$ ...	...	0·25 g.
Distilled water	...	1000 cc.

A series of tubes was prepared containing this solution with the addition of increasing percentages of lemon juice. The yeast employed was a pure

culture of a baker's yeast isolated by Dr Harden. This was grown for 24 hours in yeast water glucose and the growth was washed three times with sterile distilled water by means of the centrifuge. A loopful of a suspension of this washed yeast was put into each tube of the series, the amount being judged so that a fair proportion of drops made, according to Lindner's method, with a mapping pen on a cover slip contained one, two, or three cells each. The coverslip was sealed over a moist chamber with vaseline and, after noting the number of cells in each drop, was incubated for 24 hours at 25° and again examined for growth.

By this method it was found that no growth took place in the mineral nutrient solution unless 5 % or more of lemon juice was added. It was also noticed however that some of the tubes containing smaller amounts of lemon juice than 5 %, which had been standing in the laboratory, contained an amount of yeast appreciably larger than the amount originally inoculated. This suggested that growth did not take place as readily in the case of one or two cells in a minute drop as in a larger amount of medium. Subsequent investigation confirmed this, and the drop method was abandoned in favour of estimating the number of cells per cc. by means of a counting chamber.

In order to avoid adding any appreciable amount of "bios" with the seed yeast, it is necessary to inoculate each tube with as small a quantity of yeast as possible. Attempts to inoculate tubes by picking up a drop of distilled water containing one, two, or three cells from a coverslip by means of sterile filter paper proved unsuccessful. The method adopted was to make a light emulsion in sterile distilled water of yeast cells prepared and washed as described above. A slide was marked with several circles with a grease pencil and a drop of the emulsion transferred to the centre of each by means of a platinum loop. The drops were dried and stained and the number of cells in each counted, and by this means an emulsion was prepared which contained somewhere between 50 and 500 cells in a loopful. One loopful was then used to inoculate each tube.

A series of ten tubes was prepared with a mineral nutrient solution containing $(NH_4)_2SO_4$ with increasing percentages of lemon juice. A similar series was prepared without the $(NH_4)_2SO_4$.

Each tube contained 7 cc. of the following solution (Williams' solution without the $(NH_4)_2SO_4$, see p. 137):

Saccharose	...	20 g.
KH_2PO_4	...	2 g.
$CaCl_2$	...	0·25 g.
$MgSO_4$	...	0·25 g.
Distilled water	...	1000 cc.

The tubes containing $(NH_4)_2SO_4$ received 1·5 cc. of a solution containing 2 g. of the salt in 100 cc. The saccharose and the $(NH_4)_2SO_4$ were purified by several recrystallisations. Repeated attempts to grow the yeast in tubes of this solution with $(NH_4)_2SO_4$ inoculated with from 50 to 500 cells in the

manner described above and without the addition of "bios" failed, no growth being observed even after a lapse of several weeks.

The lemon juice was prepared by the method of Harden and Zilva [1918], namely as follows: calcium carbonate was stirred into fresh lemon juice in excess of the amount required to neutralise all free acid. Absolute alcohol to twice the volume of the original lemon juice was added, and the whole filtered. The filtrate was evaporated down almost to dryness *in vacuo* at 35°. and the residue made up to the original volume of the lemon juice with distilled water. The sample used contained 0·0868 mg. of nitrogen per cc. of which 0·0112 mg. was ammoniacal nitrogen. The desired amount of this lemon juice was added to each tube of the series which was then made up to 10 cc. with distilled water. The tubes were sterilised by steaming for 30 minutes on each of three successive days. Each was then inoculated with from 50 to 500 cells as described above, the tubes were aerated by blowing air into them through a sterile glass tube plugged with wool and having its end drawn out to a fine point, and were incubated at 25°.

The tubes were examined each day and the number of cells counted by means of a Thoma haemacytometer. At least four drops were counted from each tube and if any considerable discrepancy was found between them the counting was continued until the margin of error was reduced below 5 %. Owing to the number of tubes it was not possible to count all of them every day, but the different rates at which the yeast grew in the various tubes rendered this unnecessary. The results are set out in Table I.

Table I.

Number of tube	Percentage of lemon juice	$(NH_4)_2SO_4$	Days: growth in millions of cells per cc. 1	2	3	4	5	6	9
1	0·1	0	s.g.	s.g.	s.g.	s.g.	—	s.g.	1·6
11	0·1	+	,,	,,	,,	,,	—	1·5*	29·0†
2	0·25	0	,,	,,	,,	,,	—	1·8	1·0
12	0·25	+	,,	,,	,,	,,	—	4·3*	32·6†
3	0·5	0	,,	,,	,,	1·3	—	3·5	1·0
13	0·5	+	,,	,,	,,	1·7	—	2·1*	34·2†
4	0·75	0	,,	,,	,,	0·9	—	2·5	1·8
14	0·75	+	,,	,,	,,	1·0	—	4·9*	26·7†
5	1·0	0	,,	,,	2·8	3·1	—	2·7	6·1
15	1·0	+	,,	,,	1·1	1·4	—	4·6*	22·6†
6	2·5	0	,,	2·8	4·3	—	6·3	—	—
16	2·5	+	,,	3·1	5·5	—	21·2	—	—
7	5·0	0	,,	4·9	7·1	—	—	—	—
17	5·0	+	,,	4·8	27·4	—	—	—	—
8	7·5	0	,,	6·7	9·2	—	—	—	—
18	7·5	+	,,	21·1	19·4	—	—	—	—
9	10·0	0	,,	12·0	12·1	—	—	—	—
19	10·0	+	,,	26·5	29·4	—	—	—	—
10	15·0	0	,,	15·9	19·6	—	—	—	—
20	15·0	+	,,	24·3	40·6	—	—	—	—

S.g. means some growth but too slight to count.
* Involution forms present.
† Films formed. Accurate counting impossible owing to clumping of cells. Many involution forms.

It is well known that the growth of yeast is susceptible to slight variations of conditions which cannot be controlled absolutely, such as the degree of aeration and the formation of products of metabolism and fermentation. Further, the accuracy of the counting is affected by the smallness of the drop examined in the low counts and by the tendency of the cells to form clumps in the higher counts or in the later stages of growth, so that below 3,000,000 and above 15,000,000 cells per cc. the counts of different drops of the same sample are found to be noticeably less uniform than between those figures, and the counts made after more than six days of growth can only be regarded as approximate. In considering the results obtained regard must be paid to these facts, and account taken only of large differences in the numbers. The table shows, however, that, until the yeast reaches a concentration of somewhere in the neighbourhood of five or six million cells per cc., the rate of growth is independent of the presence or absence of $(NH_4)_2SO_4$ and depends on the concentration of the lemon juice.

It does not appear unreasonable that it should take longer for an individual cell to collect sufficient nutriment to enable it to multiply when the nutriment is dilute than when it is concentrated, and possibly the fact that cells were unable to multiply in a minute drop in dilutions that enabled growth to take place in larger quantities of medium is explained by the fact that the whole drop did not contain sufficient nutriment.

After the yeast reaches a concentration of five or six million cells per cc. it is able to continue growing freely in the $(NH_4)_2SO_4$ tubes. Further it was observed that after six days involution forms, as described by Will [1895] in his investigation of film formation, begin to appear in all tubes containing $(NH_4)_2SO_4$ where the growth had not reached the critical point of five or six million cells per cc., and by the ninth day there was a heavy growth of film yeast on all these tubes. All the remaining tubes after a lapse of three weeks showed only very rare involution forms and no film formation, but most of the cells were highly refractile with many large vacuoles and granules and a thick membrane, similar in appearance to the permanent cells described by Will. It would appear, then, that after an interval of six or seven days the cells in smaller concentrations than five or six million per cc. are able to adapt themselves to the use of $(NH_4)_2SO_4$ and that film formation results.

A similar series of tubes was prepared with increasing percentages of aqueous yeast extract instead of lemon juice. This was prepared by Osborne and Wakeman's method [1919]; about 250 g. of wet brewer's yeast was washed and boiled for two minutes with 1 litre of distilled water containing 0·01 % of acetic acid and the liquid was separated off in the centrifuge. The solid residue was again heated with 500 cc. of 0·01 % acetic acid and the extracts united and concentrated to 500 cc. This extract contained 2·856 mg. of nitrogen per cc., of which 0·2273 was ammoniacal nitrogen. As this is rather more than 30 times the amount contained in the lemon juice, the amounts added to the various tubes of the yeast extract series were much smaller than in the lemon juice series. The results are set out in Table II.

Table II.

Number of tube	Percentage of yeast extract	$(NH_4)_2SO_4$	Days: growth in millions of cells per cc. 1	2	3	4	5	6	11
21	0·01	0	s.g.	s.g.	s.g.	s.g.	—	—	3·1
31	0·01	+	,,	,,	,,	,,	—	—	10·6*
22	0·02	0	,,	,,	,,	,,	—	—	3·0
32	0·02	+	,,	,,	,,	,,	—	—	10·5*
23	0·05	0	,,	,,	,,	,,	—	—	4·0
33	0·05	+	,,	,,	,,	,,	—	—	15·0*
24	0·1	0	,,	,,	,,	,,	—	1·6	10·0*
34	0·1	+	,,	,,	,,	,,	—	15·1*	20·0*
25	0·15	0	,,	,,	,,	6·1*	—	10·0*	—
35	0·15	+	,,	,,	,,	3·6	—	11·5*	—
26	0·2	0	,,	,,	0·5	3·6	5·1	—	—
36	0·2	+	,,	,,	0·5	3·7	15·5	—	—
27	0·5	0	,,	4·9	7·6	—	—	—	—
37	0·5	+	,,	4·4	23·7	—	—	—	—
28	1·0	0	,,	5·3	14·5	—	—	—	—
38	1·0	+	,,	11·8	39·5	—	—	—	—
29	2·0	0	,,	13·6	27·2	—	—	—	—
39	2·0	+	,,	19·9	43·0	—	—	—	—
30	3·0	0	,, } Very heavy growth. Not counted.						
40	3·0	+	,, }						

S.g. means some growth but too slight to count.
* Involution forms present. Very slight film formation.

On comparing the results obtained with the yeast extract with those obtained with the lemon juice it will be seen that their general trend is similar, and that the yeast extract is approximately ten times as effective as the lemon juice although its nitrogen content is more than 30 times as great. The results obtained are somewhat less uniform than those obtained with the lemon juice, and tubes Nos. 24 and 25 fall out of line on the 11th and 4th days respectively. Film formation does not occur so readily, only very slight traces being present in tubes Nos. 31, 32, 33, 34 and 35 by the 11th day.

These differences may be due to differences in the nature of the nitrogen in the two substances under examination or to the presence of toxic substances in the yeast extract which are absent from the lemon juice. The yeast experiment, however, affords confirmation of the fact that the rate of growth is at first independent of the presence or absence of $(NH_4)_2SO_4$ and depends on the concentration of the "bios" until the yeast has reached a concentration of somewhere in the neighbourhood of five or six million cells per cc., after which it proceeds further in the presence of the $(NH_4)_2SO_4$.

General Conclusions.

The suggestion that "bios" may be of the same nature as the vitamins, which are widely held to be necessary for the nutrition of higher animals, and the further suggestion that it may be identical with one of the recognised vitamins, open up possibilities for investigating the subject of vitamins which are particularly attractive, owing to the ease with which a biological process

can be studied in a yeast as compared with a similar study in the case of a more complex organism. Before we can proceed to investigate the general question of vitamins by such methods it is necessary, firstly, to be certain that "bios" is actually a vitamin, *i.e.* a substance whose presence in the food in relatively small quantities is necessary to enable the remainder of the foodstuffs to be properly assimilated and utilised by the organism, and secondly that the substance in question is identical with one of the recognised vitamins. The present investigation points to the fact that "bios" does not enable the yeast to assimilate $(NH_4)_2SO_4$ simply by its presence or by being consumed at the same time, but that the yeast grows solely at the expense of the "bios" until it reaches a certain degree of concentration, and after that it is able to use the $(NH_4)_2SO_4$. No explanation is offered of this phenomenon which requires further investigation.

I wish to thank Prof. A. Harden, who suggested the investigation, for his advice and assistance in carrying it out.

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XX. THE FUNCTION OF PHOSPHATES IN THE OXIDATION OF GLUCOSE BY HYDROGEN PEROXIDE.

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The conditions under which the oxidation of glucose by H_2O_2 can take place in aqueous solution have been studied by W. Löb and his co-workers [1910, 1911, 1912, 1915]. He believes that the oxidation depends on the hydroxyl ion concentration of the solution, and further that phosphate ions exercise a specific accelerating effect on the reaction. He was unable to produce any oxidation of glucose by H_2O_2 in presence of water alone or when other substances were substituted for phosphates.

For instance neither Sörensen's mixtures of glycocol and NaOH nor of borate and HCl could be used to replace the phosphate mixture. Witzemann [1920] confirmed Löb's observations and agreed that phosphate mixtures exercise a specific effect. He was unable to produce the same effect with mixtures of $NaHCO_3$ and Na_2CO_3 as with phosphate mixtures.

Witzeman [1920] suggested that the phosphates might produce an intermediate compound with glucose of the same nature as the hexosephosphate which has been shown by Harden and Young [1910] to be produced during alcoholic fermentation, and which appears from the work of Embden and others to be connected with the production of lactic acid in muscle.

Witzemann was unable to detect the formation of any compound of glucose and phosphate in absence of H_2O_2.

As more exact knowledge of the mechanism of this reaction is desirable in view of the part played by phosphate in alcoholic fermentation, further experiments were made to try and ascertain whether an intermediate compound of the nature of a phosphoric ester of glucose was formed during this oxidation. For this purpose free phosphate was estimated at intervals in a mixture similar to one of those used by Witzemann for oxidation of glucose by H_2O_2 at 37°, both by the method of Schmitz [1906] and by the ordinary magnesium citrate method. No change in the amount of free phosphate was observed.

Besides the possibility of the formation of an intermediate compound between phosphate and glucose there are at least two other possible explanations of the effect produced by phosphate in this reaction:

(1) Phosphate may act as a peroxidase, thus rendering possible the oxidation of glucose by H_2O_2. If this were so phosphate might act in the same way towards mixtures of H_2O_2 and benzidine or guaiacum. No evidence could be obtained of such an action, but it does not follow from this negative result that no such action occurs with H_2O_2 and glucose.

(2) The phosphate mixtures may simply act as buffer substances, the maintenance of the P_H within certain points being essential. If this hypothesis is correct it should be possible to replace the phosphate mixtures by other substances, provided the P_H be maintained within the same limits.

We have carried out experiments on the oxidation of glucose by H_2O_2 with solutions the P_H of which was never over 7·3 and find that glucose is oxidised by H_2O_2 in presence of 0·125 *M* $NaHCO_3$ saturated with CO_2; or 0·25 *M* sodium arsenate saturated with CO_2; or 0·25 *M* sodium acetate.

The phosphate ion cannot therefore be regarded as playing a specific part in the reaction. It appears more likely that the buffer action of the salts employed is the important factor, whether as providing and maintaining the most suitable conditions for oxidation or as protecting the H_2O_2 from too rapid decomposition. In this connection the experiment of Witzemann [1920, p. 12, sec. 7] with a mixture of sodium carbonate and bicarbonate should be referred to. This shows that in presence of these salts, at a P_H slightly over 9·3, H_2O_2 is rapidly decomposed and little oxidation takes place, whereas in our experiment in presence of 0·125 *M* $NaHCO_3$ saturated with CO_2 (P_H 6·8) a considerable amount of oxidation is produced.

Experiments were accordingly made roughly to test the stability of H_2O_2 in solutions the P_H of which was maintained at various levels by the use of buffer solutions. The rate of decomposition was found to increase with rise of alkalinity. In those cases in which the buffer solutions consisted of phosphate mixtures the rate of decomposition of the H_2O_2 was not so rapid as in the corresponding experiments with other buffer solutions; so that in this sense the phosphate ion may be said to have a specific action.

In the experiments of both Löb and Witzemann the P_H of the solutions was only carefully measured in those experiments in which Sörensen mixtures were used. In most if not all of the experiments in which the effects of other substances were compared to that of phosphate mixtures the reaction of the solution was tested with litmus paper only. The glycocol + NaOH and borate + HCl mixtures used are only efficient buffers between P_H 8·0 and 10·2, and at these higher degrees of alkalinity the H_2O_2 is rapidly decomposed. The same applies to the $NaHCO_3 + Na_2CO_3$ mixture used by Witzemann which must have been much more alkaline than the phosphate mixture.

The experiments in which he compared the effect of Na_2CO_3 and NaOH were not carried out at the same P_H. The reaction of the solution in the latter case was suitable for the oxidation, whereas the alkalinity of the solution used in the former case was too high.

Experimental.

I. *Experiments to determine if mixtures of* Na_2HPO_4 *and* NaH_2PO_4 *will act as a peroxidase towards* H_2O_2 *and benzidine or guaiacum.*

Phosphate mixtures of P_H 9·0, 7·4, 6·9, 6·6, 6·2, 4·8 were tested with H_2O_2 and benzidine or guaiacum. No action was observed in any of the experiments.

To test whether the optimum P_H for the action of a peroxidase lies within the above range, similar experiments were made with the same phosphate mixtures, H_2O_2 and guaiacum with the addition of potato juice. The maximum effect was produced at room temperature from P_H 7·4 to 6·9. A similar result was obtained when benzidine was substituted for guaiacum.

II. *Oxidation of glucose by* H_2O_2 *in presence of various buffer mixtures.*

Seven flasks were made up as follows:

	Glucose g.	$NaHCO_3$ g.	Na_2HAsO_4. 12 H_2O g.	$NaC_2H_3O_2$. 3 H_2O g.	K_2HPO_4 g.	KH_2PO_4 g.	5·7 % sol. H_2O_2 cc.	
1	8·75	2·6	—	—	—	—	62	to 250 cc. with distilled water
2	8·75	—	25·1	—	—	—	62	
3	8·75	—	—	8·5	—	—	62	
4	8·75	—	—	—	8·8	2·2	62	
5	11·25	2·6	—	—	—	—	—	
6	11·25	—	25·1	—	—	—	—	
7	11·25	—	—	8·5	—	—	—	

In Nos. 1, 2, 5, 6 the solution was saturated with CO_2.

The P_H of each solution was estimated colorimetrically, and the glucose estimated by Bertrand's method and by the polarimeter.

All the flasks were then kept in the incubator at 37° for 24 hours and the P_H and glucose again estimated in each. The results are shown below:

	At start of experiment glucose g. per 100 cc.			After 24 hours			Apparent loss of glucose in g. per 100 cc.	
	P_H	Bertrand A	Polarimeter B	P	A	B	by A	by B
1	6·8	4·16	3·50	6·7–6·8	3·39	2·99	0·77	0·51
2	6·8	3·96	3·55	6·7–6·8	2·96	2·38	1·00	1·17
3	6·8–7·0	3·64	3·54	5·2	3·01	2·50	0·63	0·53
4	7·0	3·65	3·20	5·6–5·8	2·24	1·85	1·41	1·35
5	6·8	—	4·55	7·6	—	4·55	—	0
6	6·8	—	4·50	7·0	—	4·50	—	0
7	7·3	—	4·25	7·3	—	4·25	—	0

Note. The sample of glucose employed for these experiments showed a 5 % higher content of glucose as estimated by Bertrand's method than when the polarimeter and $[\alpha]_D$ 52·5° were used. This, and the fact that the Bertrand estimations of glucose at the start of the experiment were carried out before the polarimeter readings were made, may account for the discrepancies between the two sets of figures shown above.

Only traces of H_2O_2 remained in 1, 2 and 4 after 24 hours in the incubator.

No. 3 still contained a considerable amount of H_2O_2 and the action was allowed to proceed for a second period of 24 hours. After this the P_H was

found to be 4·6–4·8 and glucose estimated polarimetrically amounted to 1·76 g. per 100 cc.: a total apparent loss of glucose of 1·78 g. per 100 cc.

These experiments show that in absence of H_2O_2 no loss of glucose has apparently taken place (Nos. 5, 6, 7). Whereas in presence of H_2O_2 (Nos. 1, 2, 3, 4) an apparent loss of glucose has occurred in every case, greatest in the two solutions containing phosphates and arsenates, but considerable in the other two solutions.

III. *Effect of change of* P_H *on the stability of* H_2O_2.

All solutions contained 5·0 cc. of 5·7 % H_2O_2 per 100 cc. solution. The H_2O_2 was estimated at the start and again after 24 hours and 48 hours at 37° by titration with standard permanganate.

No.	Mixture	P_H	% H_2O_2 lost after 24 hours	% H_2O_2 lost after 48 hours
1	HCl	1·0	—	1
2	Sörensen's glycocol + HCl ...	1·9	1	2·1
3	Na acetate + acetic acid ...	4·6	2	2·1
4	Phosphates	6·8	2	4·2
5	,, ...	7·1*	4·9	7·5 (43 hours)
6	,, ...	7·8*	6·1	7·4 (43 hours)
7	,, ...	8·0*	7·5	13·1
8	Palitzsch's borax + boric acid	6·9	20·2	36·1
9	,, ,, ,,	7·1	20·2	37·2
10	,, ,, ,,	7·7	22·3	39·3
11	,, ,, ,,	7·9	37·2	59·5
12	,, ,, ,,	8·2	55·3	74·4
13	,, ,, ,,	8·4	65·9	84·0
14	,, ,, ,,	8·6	65·9	84·0
15	,, ,, ,,	8·9	67·0	85·1
16	Sörensen's borate + HCl ...	8·8	—	78·5
17	,, + NaOH ...	9·2	—	99·0
18	,, ,, ...	12·8	100	—

* In these cases the concentration of the hydrogen peroxide used was 5 % instead of 5·7 %.

IV. *The* P_H *of some of the solutions tested by Witzemann.*

A solution was made up as described by Witzemann [1920, p. 12, sec. 7]: 2·43 g. $Na_2CO_3 . 10H_2O$; 0·72 g. $NaHCO_3$; 35 cc. H_2O; 20 cc. (0·2 g.) glucose solution and 20 cc. of 3 % H_2O_2.

The P_H, estimated colorimetrically, was over 9·3. At this degree of alkalinity H_2O_2 is rapidly decomposed and it is therefore not surprising that little oxidation took place.

To compare the effect of NaOH and Na_2CO_3 Witzemann [1920, p. 14, sec. 9 (2) and sec. 10 (2)] prepared solutions as follows:

P. 14, sec. 9: 32·0 cc. 0·33 *M* Na_2HPO_4; 3·0 cc. H_2O; 20·0 cc. 1 % glucose; 20·0 cc. 3 % H_2O_2; 0·61 g. $Na_2CO_3 . 10H_2O$;

and sec. 10 (3) 25·6 cc. 0·33 *M* Na_2HPO_4; 6·4 cc. 0·33 *M* NaH_2PO_4; 20·0 cc. 1 % glucose; 20·0 cc. 3 % H_2O_2; 3 cc. H_2O; 0·1714 g. NaOH.

The P_H of the former was 9·3–9·6 and of the latter 7·1. The amount of oxidation was greater in the second than in the first case and Witzemann argues that NaOH exercises a less harmful effect on the reaction than does Na_2CO_3.

It does not seem that this inference can fairly be drawn unless the experiments are carried out under the same conditions of P_H. The acidity of the H_2O_2 solution used by Witzemann is not stated in his paper. It is therefore not possible to say what was the exact P_H in his experiments. But in any case the alkalinity of the solutions used in experiments 7 and 9 (2) must have been much higher than in experiment 10 (3).

Summary.

1. The oxidation of glucose by H_2O_2 takes place in presence of the following buffer substances: $NaHCO_3 + CO_2$; $Na_2HAsO_4 + NaH_2AsO_4$; $NaC_2H_3O_2$; $K_2HPO_4 + KH_2PO_4$, provided the P_H does not rise much above 7·3.

2. The stability of H_2O_2 in aqueous solution is increased by the presence of phosphates which do not however show a specific action in other respects.

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XXI. VITAMIN REQUIREMENTS OF *DROSOPHILA*. I. VITAMINS *B* AND *C*.

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Loeb [1915] ascertained the fact that flies of the genus *Drosophila* could be reared on a culture medium containing as mineral constituents only K, Mg, PO_4 and SO_4 ions. In these experiments micro-organisms were not excluded, and the growing larvae undoubtedly lived at the expense of these. Loeb also found that when Na was substituted for K no growth took place and concluded that K was essential for the development of the insect. It is however possible that the absence of K stopped the growth of the micro-organisms and thus deprived the larvae of nitrogen in an available form. Loeb's experiments therefore only prove that the mineral constituents named above are adequate, but not that they are essential.

Subsequently Loeb and Northrop [1916] succeeded in sterilising the eggs of *Drosophila* by exposure for 6–7 minutes to 0·1 % aqueous $HgCl_2$ or a saturated alcoholic solution of the same salt and found that the larvae hatched out from the few eggs which survived the disinfection were sterile. They ascertained that these sterile larvae could not be reared on sterile media consisting only of purified proteins, carbohydrates, fats and salts, but that when yeast killed by heat was added, normal development occurred. Alcoholic extracts of yeast were however inactive. Northrop [1917] pursued the subject further and found that the addition of caseinogen and similar substances along with cane sugar to yeast greatly increased the number of flies which could be reared at the expense of a definite amount of yeast and concluded that they acted as foodstuffs when properly supplemented by yeast. Similar results were obtained by the addition of kidney, liver and pancreas from the dog, but many other organs of the dog were inactive. The author was inclined to interpret these results in the sense that yeast supplies one or more accessory substances, necessary for the growth of the organism.

The question was again investigated by Baumberger [1919] who found that the pupae and eggs of flies reared in presence of a pure culture of yeast could readily be sterilised by immersion for 20 minutes in 85 % alcohol. The sterile larvae obtained from such eggs could easily be reared in sterile culture media provided the latter contained killed yeast or nucleo-protein prepared from yeast. Baumberger came to the conclusion that the essential requisite

was a sufficient concentration of yeast nucleo-protein and that accessory food factors were not concerned.

The following experiments were instituted with the object of ascertaining more definitely whether any of the recognised vitamins are essential for the nutrition of these flies. The subject is of general interest as regards the extent to which the need for accessory food factors is distributed in the animal kingdom. Special interest attaches to the possibility of introducing a simplified technique by which results could be obtained more rapidly and economically than by the tedious and costly feeding experiments which are at present necessary.

Technique of the Experiments.

It was soon found that the eggs could be sterilised much more readily and certainly than the pupae and this method was always employed.

Following Baumberger's instructions the flies were reared in banana-agar (approximately equal parts of mashed banana and 1·5 % water-agar) which had been autoclaved and subsequently inoculated with yeast (*S. cerevisiae*). After a few sub-cultures on this medium the flies were found to be free from bacteria, both anaerobic and aerobic, and the pure stock was readily maintained by occasionally passing a few flies into a fresh banana-agar-yeast tube.

When eggs were desired for experimental purposes, freshly emerged flies were transferred to a special apparatus. This consisted of an inverted wide necked bottle, standing in a Petri dish and having a strip of moistened filter paper on the wall. A small circular glass dish with vertical walls was also placed on the Petri dish, so as to be covered by the inverted bottle. The walls of this dish were lined with hardened filter paper moistened with water. The bottle was in turn covered by an inverted cylindrical tinned iron can and the whole apparatus was autoclaved at 120° for half-an-hour. A few cc. of a 24–48 hour culture of yeast in yeast extract containing glucose were then placed in the small dish, the flies introduced into the inverted bottle and the whole apparatus placed in an incubator at 30°. Under these conditions the eggs were laid within 24–48 hours almost exclusively on the hardened filter paper lining the small circular glass dish containing the yeast culture. The strips of paper were removed, placed on a sterile slide under a dissecting microscope and the eggs picked off singly by means of sterilised dissecting needles and placed in 85 % alcohol as directed by Baumberger. They were then divided up into collections of the number required for each tube by means of a sterile pipette provided with a rubber teat, and were finally, after half-an-hour's exposure to the alcohol, sucked up into the pipette and delivered into the appropriate tube of medium. It was found most convenient to place 20 eggs in each test tube ($6'' \times \frac{3}{4}''$) containing 5–5·5 cc. of medium in the form of an agar slope.

The agar jelly in the medium tube must be so soft that the larvae can readily penetrate it in their search for food. As a rule 3 cc. of $1\frac{1}{2}$ % agar

were added to 2 cc. of medium and the whole then steamed for 20 minutes on three successive days, and cooled in the form of slopes.

The inoculated tubes were incubated at 30° and examined daily, note being made of the number hatched, the general progress and the time of appearance of pupae and flies.

At the close of each experiment the tubes were tested for sterility by making cultures on beef broth-peptone-agar and incubating at 37°. Contamination by yeast usually showed itself in the experimental tube, but was when necessary tested for by culture on beer wort agar at 25°.

Diet.

A basal diet consisting of

Caseinogen (purified)	0·15 g. per tube
Starch and salts[1] (94 % starch and 6 % salts)	0·05 g. „
Cane sugar	0·1 g. „

was employed, to which various additions of butter-fat (vitamin *A*) yeast-extract (vitamin *B*) and lemon juice freed from citric acid (vitamin *C*) were made as required, 2 cc. of liquid in all being added, and then 3 cc. of 1½ % agar. The caseinogen had been extracted with alcohol and light petroleum or in some cases heated at 120° for many hours and repeatedly stirred.

Results.

The experiments were directed in the first instance to ascertaining which, if any, of the three recognised accessory food factors are necessary for the growth and metamorphoses of these insects.

The results, which are summarised below, showed definitely that:

(1) in the presence of butter-fat and yeast extract (vitamins *A* and *B*) growth occurred irrespective of the presence or absence of 1 cc. of lemon juice (vitamin *C*);

(2) in the presence of butter-fat (vitamin *A*) growth occurred in presence of 1 cc. of yeast extract (vitamin *B*), but not in its absence, this result being independent of the presence or absence of lemon juice;

(3) in the presence of 1 cc. of yeast extract (vitamin *B*), with or without 1 cc. of lemon juice (vitamin *C*), growth occurred in the presence of two drops of butter-fat, but not in its absence.

In the following summary only results of experiments in which the tubes remained sterile are quoted.

	Additions to basal diet						
	Butter-fat	Yeast extract	Lemon juice	Tubes inoculated	Eggs hatched	Pupae formed	Flies emerged
1	−	+	+	10	100	0	0
2	+	+	−	11	115	92	63
3	+	−	+	10	57	0	0
4	+	+	+	10	68	48	42
5	−	+	−	2	19	0	0
6	−	−	−	2	16	0	0

[1] As used in this laboratory for rats.

Control experiments were carried out to ascertain whether the yeast extract alone was capable of supplying sufficient nitrogen for the growth of the larvae, but with entirely negative results. This was done by omitting the caseinogen from mixtures 2 and 4, which gave positive results in its presence. Even the addition of 2 cc. of the yeast extract gave entirely negative results in the absence of the caseinogen.

Other sources of vitamin B.

As flies of this group are specially associated with yeast, which probably forms the chief food of the larvae in their natural condition, it was thought that in order to prove the necessity for vitamin *B*, it would be essential to show that it could be supplied from other sources.

The first experiments were made with milk, but without success. Recourse was then had to wheat germ, the alcoholic (80 %) extract of which is known to contain a considerable amount of vitamin *B*.

50 g. of wheat germ were heated on the water-bath for one hour with 200 cc. of 80 % alcohol, pressed out and the clear liquid evaporated to dryness at 50°. The residue was shaken up with 50 cc. of water and filtered, the clear filtrate being used. This contained 10·47 g. total solids, 0·21 g. mineral matter, and 0·196 g. N per 100 cc.

Tubes were made up with 2, 1 and 0·5 cc. of this extract in addition to the usual basal diet of caseinogen, starch, salts, cane sugar and butter-fat. Some difficulty was experienced in adjusting the stiffness of the agar jelly, but finally 0·5 cc. H_2O and 3 cc. of 1·25 % agar were added to each tube, so that the total volume was 5·5 cc. as shown below.

	Caseinogen, starch and salts g.	Cane sugar g.	Butter-fat	Wheat germ extract cc.	H_2O cc.	1·25 % agar cc.
7	0·2	0·1	2 drops	2	0·5	3
8	0·2	0·1	,,	1	1·5	3
9	0·2	0·1	,,	0·5	2·0	3
10	Starch and salts 0·05	0·1	,,	2	0·5	3

The following are the summarised results of three quite independent and consistent experiments:

	Wheat germ extract	Eggs known to have hatched	Pupae	Flies
11	2	34	18	9
12	1	56	24	13
13	0·5	39	0	0
14	2 cc. as sole source of N	9	0	0

The experiments were somewhat hindered by the hot weather prevailing at the time, which caused unduly rapid drying of the tubes, but they show quite conclusively that, like yeast extract, wheat germ extract is capable of supplying some factor necessary for the growth and metamorphosis of the fly.

It is at the same time obvious that the wheat germ extract (1 cc. of which corresponds with 1 g. of wheat germ) is much less efficacious than the yeast

extract (1 cc. of which corresponds with 0·5 g. yeast) and this is in accordance with what is known of the vitamin *B* content of these two materials, 1 g. of yeast being approximately equivalent to 3·6 of wheat germ.

Taken together the experiments with yeast extract and wheat germ extract leave little doubt that these insects require vitamin *B* for their development, thus confirming Northrop's view. Experiments are in progress on the amount of yeast extract required and the effect of varying concentrations on the duration of the period of development.

The Fat-soluble Factor (Vitamin *A*).

The striking fact that in presence of 1 cc. yeast extract growth occurred in the presence of two drops of clarified butter-fat, but not in its absence, may be taken to show either that a fat of some kind is necessary or that the fat-soluble factor is required or that both are required. To decide between these possibilities it is necessary to ascertain whether or not growth occurs in presence of a fat devoid of vitamin *A*. Attempts of this kind are being made, but no definite result has yet been obtained. The preliminary results however indicate that the fly is certainly able to develop in the presence of very small amounts of the vitamin if not in its entire absence.

Summary.

The complete development of *Drosophila* requires the presence of vitamin *B* but not of vitamin *C*.

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